Haemin-induced haemoxygenase (HO-1) expression in ischaemia / reperfusion injury of the rat heart

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HAEMIN-INDUCED HAEMOXYGENASE (HO-1) EXPRESSION IN ISCHAEMIA / REPERFUSION INJURY OF THE RAT HEART

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Abstract

Haemoxygenase-1 (HO-1) is an important enzyme involved in the breakdown of pro-oxidant haem, producing carbon monoxide (CO), free iron and biliverdin (subsequently converted to bilirubin). These mediators appear to have important roles; CO is a vasodilator and an inhibitor of VSMC proliferation / apoptosis; Bilirubin is a potent anti-oxidant; free iron produces ROS and induces ferritin expression.

HO-1 was induced using haemin and inhibited using tin protoporphyrin (SnPP). The effect of HO-1 on ischaemia/reperfusion injury was determined in the isolated rat heart perfused at either a constant-flow of 10 ml/min or a constant-pressure of 70 mmHg over a range of ischaemic periods (20-40 min). The effect of haemin pre-treatment was also determined in other vascular beds such as the perfused kidney and perfused mesenteric vascular beds.

At constant-flow; haemin pre-treatment significantly improved the recovery of cardiac function (20min ischaemia), produced a vasodilator response and increased tissue bilirubin. The recovery was dependent on cycloxygenase (COX) products and NO was involved in the dilator response. At constant-pressure; haemin pre-treatment did not aid protection (as recovery was ~80% in both treatment groups) but was accompanied by a vasoconstrictor effect pre-ischaemia. Increased ischaemic insult abolished recovery in both perfusion models. Desferrioxamine restored the recovery of cardiac function at constant-pressure. At a constant pressure of 130mmHg haemin-induced recovery from I/R and also produced a vasoconstrictor effect. The vasoconstrictor response observed at constant-pressure was not related to endogenous autoregulatory mechanisms. Haemin did not affect the vascular responsiveness in the perfused kidney and mesentery.

In conclusion, haemin pre-treatment can confer protection from I/R in both the constant-flow and constant-pressure perfused heart due to the anti-oxidant effects of bilirubin and the vasodilatory effects of CO. However the response appears to involve more complex mechanisms, including the release of other vasoactive mediators (COX products & NO), the pro-oxidant effects of iron, the method of perfusion and the pressure of the system.

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List of Abbreviations

ACh	Acetylcholine
CAT	Catalase
CO	Carbon monoxide
COX	Cycloxygenase
CFR	Coronary flow rate
CPP	Coronary perfusion pressure
CrMP	Chromium mesoporphyrin
DFO	Desferrioxamine
ET-1	Endothelin-1
GSH	Glutathione
HO-1	Haemoxygenase-1
HO-2	Haemoxygenase-2
HO-3	Haemoxygenase-3
HR	Heart rate
HSP	Heat shock protein
HOCl	Hypochlorous acid
5-HT	5-hydroxytryptamine
ICAM-1	Intercellular adhesion molecule-1
IPC	Ischaemic preconditioning
I/R	Ischaemia / Reperfusion
LTB_4	Leukotriene B_4
LDL	Low-density lipoprotein
LVDT	Left ventricular developed tension
MPO	Myeloperoxidase
NA	Noradrenaline
NO	Nitric oxide
NOS	Nitric oxide synthase
PE	Phenylephrine
PKC	Protein kinase C
PLA_2	Phospholipase A_2
PAF	Platelet activating factor
ROS	Reactive oxygen species

SIN-1	3-morpholinosyalnomine
SOD	Superoxide dismutase
SNAP	S-nitro-N-acetyl penicillamine
SnPP	Tin Protoporphyrin
SNP	Sodium nitroprusside
SX6C	Sarafotoxin-6C
TxA_2	Thromboxane A_2
VEGF	Vascular endothelial growth factor
VF	Ventricular fibrillation
ZnPP	Zinc protoporphyrin

CHAPTER 1:

INTRODUCTION

Chapter 1: Introduction

1.1 Ischaemia-Reperfusion injury of the heart.

Myocardial ischaemia-reperfusion (I/R) occurs when a temporary coronary occlusion results in the absence of fluid perfusion through a specific tissue region and induces a temporary hypoxia. I/R injury is a common occurrence in a number of pathophysiological situations such as coronary heart disease, myocardial infarction and coronary bypass surgery. Myocyte damage may be reversible or irreversible dependent upon the length of ischaemic insult. Reversible damage is observed as a mitochondrial swelling, a loss of dense mitochondrial granules, and an absence of granular flocculent densities within the mitochondria. Irreversibly damaged myocytes have shrunken nuclei, formation of sarcolemmal blebs, myofibril fragmentation and sarcoplasmic reticulum disruption (Virmani et al., 1990). After short periods of ischaemia (5-10 min), there is generally complete recovery of function, but recovery of function decreases as the ischaemic period increases to 20-60 min (Taegtmayer et al., 1997).

It is now widely agreed that the majority of injury received as a result of I/R occurs during the reperfusion phase of the insult, since short periods of reperfusion do not produce injury, while longer periods of reperfusion are associated with a progressive injury (Virmani et al., 1990). For example, during ischaemia-induced injury there is an increase in platelet activation and altered cardiac metabolism, demonstrated by thrombosis, vasoconstriction and ATP loss (Diagram 1). The use of preventative treatment has a similar outcome whether initiated upon reperfusion or during the pre-ischaemic period (Grisham et al., 1989). Furthermore, there must be molecular oxygen present during reperfusion to induce the tissue injury associated with I/R injury (Korthuis et al., 1989), and for recovery from I/R to occur. Thus, as indicated in diagram 1, reoxygenation is required for neutrophil recruitment and reactive oxygen species (ROS) generation. Therefore, the resultant injury post-I/R occurs as a result of a combination of direct ischaemic and reperfusion-enhanced injury.

There are a number of structural and functional changes in response to I/R. For example; I/R has a profound effect on the endothelium, as intercellular junctions are disrupted producing partial or complete detachment of the endothelium from the sub-endothelium. There is an increase in both intracellular and extracellular oedema due to a reduction in intact endothelium. Further pathologic changes observed include abnormalities in vasomotor function, coagulation, vascular permeability, inflammatory responses and intimal proliferative responses (Verrier, 1996), and increased vascular resistance producing a reduced blood flow (Quillen et al., 1990). A number of vasoactive mediators are released in response to tissue damage (see Diagram 1), these include; prostaglandins and thromboxanes, nitric oxide (NO), ROS, smooth muscle derived relaxing factors, catecholamines and products of the renin-angiotensin system (Verrier, 1996). The cumulative effect of these tissue responses is the impairment of coronary vasorelaxation or enhancement of vasoconstriction.

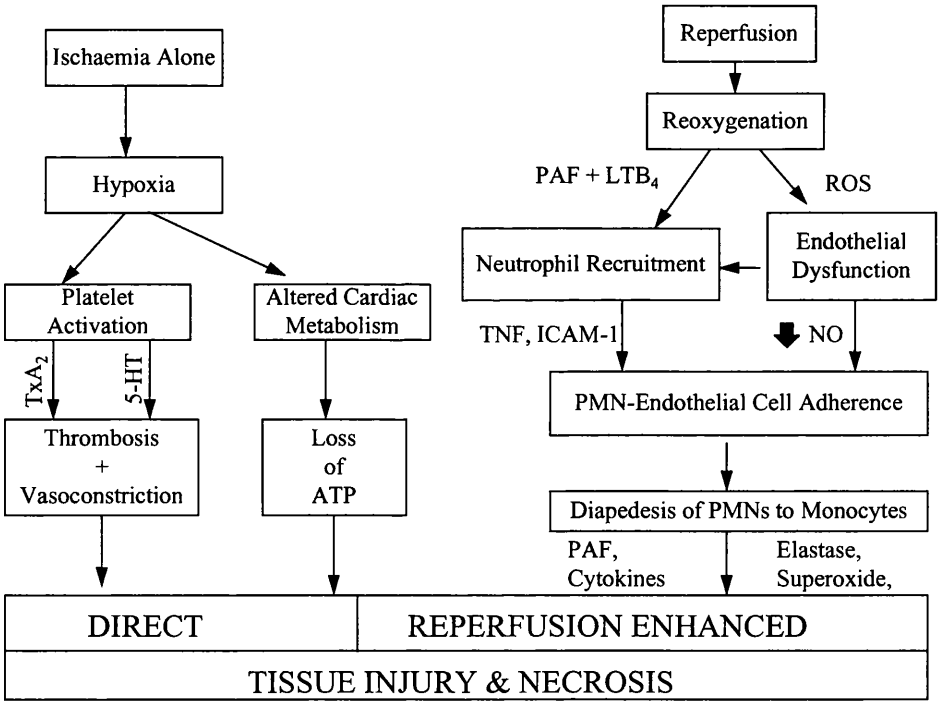


Diagram 1: The mechanisms involved in I/R injury in both ischaemia alone and reperfusion enhanced pathways (Lefer & Lefer, 1993).

Thromboxane A_2 (TxA_2), 5-hydroxytryptamine (5-HT), platelet activating factor (PAF), intercellular adhesion molecule-1 (ICAM-1), reactive oxygen species (ROS) and leukotriene B_4 (LTB_4).

One of the potentially serious side effects from I/R is the development of reperfusion-induced ventricular arrhythmias. This disruption in cardiac function may be life threatening and can be a cause of sudden cardiac death in patients. Upon restoration of coronary flow after myocardial ischaemia, there is an increased incidence of reperfusion-induced arrhythmias such as ventricular fibrillation (VF) and ventricular tachycardia (Tosaki et al., 1993). Reperfusion arrhythmias may be avoided by the administration of calcium antagonists, which have been demonstrated to suppress reperfusion-induced VF (Farkas et al., 1999). The calcium antagonist verapamil has an increased selectivity in the ischaemic myocardium due to the increase in extracellular K^+ concentration during acute ischaemia (Curtis & Walker, 1986). The pathological changes after I/R thought to be mainly responsible for the induction of arrhythmias involve the increase in levels of substrates involved in the modulation of conductance of gap junctions, such as Ca^{2+}, Mg^{2+}, protons and lipid metabolites. In combination with decreased ATP levels, the increase in the levels of these mediators produces poor gap junction conductance and increased intercellular resistance. The increasing gap junction resistance is accompanied by cell-to-cell uncoupling, activation delay and conductance block (Smith et al., 1995).

Catecholamines are particularly important in the regulation of normal cardiac function. In the heart, stimulation of β-adrenoceptors results in an increase in the rate and force of contraction, whereas activation of α-adrenoceptors can produce vasoconstriction. Therefore, modulation of catecholamine metabolism or signalling may be important in determining the outcome or severity of the injury produced in response to I/R. In particular, during acute ischaemic insult of >15min duration, local noradrenaline concentrations are increased and this in combination with an enhanced responsiveness of myocytes to catecholamines, increases the occurrence of I/R-induced arrhythmias (Schomig et al., 1991). This is confirmed in a study by Lameris et al., (2000), where the release of noradrenaline, adrenaline and dopamine progressively increased in the myocardial interstitial fluid during ischaemia. In addition, after regional occlusion of the rat coronary artery, ischaemic myocardial noradrenaline content is significantly reduced as a result of increased release (Holmsgren et al., 1981).

The release of catecholamines during the first 5-10 min of ischaemia is closely regulated by adenosine (Headrick & Willis, 1989), which inhibits the catecholamine-induced activation of adenylate cyclase activity (Stangl et al., 1999). Increased catecholamine release appears to counteract various cardiodepressant mediators released in response to I/R (Stangl et al., 1999), thereby increasing myocardial oxygen demand and increasing the severity of ischaemic insult. Excess catecholamine levels can produce cellular necrosis via β-adrenoceptor activation resulting in intracellular Ca^{2+} overload and lipolysis (Opie et al., 1979). The role of adrenoceptors in the progression of I/R has been extensively studied. For example, the activation of α1-adrenoceptors appears to be important in the formation of ventricular arrhythmias that occur during I/R. (Sheridan et al., 1980). In contrast, β-adrenoceptor receptor density is increased in ischaemic myocardium during the progression of myocardial infarction (Maisel et al., 1985). This was also demonstrated in the guinea-pig heart exposed to global ischaemia, where increased β1-adrenoceptor density and increased adenylate cyclase activity was present after 30min ischaemia (Thandroyen et al., 1990).

1.2 Mechanisms of post-ischaemic tissue injury

1.2.1 The role of reactive oxygen species

The most significant ROS involved in I/R-induced tissue damage are the superoxide anion radical, hydrogen peroxide (H_2O_2), and the hydroxyl radical (Diagram 2). The importance of oxygen for the induction of tissue injury during reperfusion may be due to the requirement of oxygen for the formation of ROS. ROS are involved in the peroxidation of various lipid components of cell membranes. These changes can result in the elimination or interference of a number of cellular functions such as, structural/contractile/transport proteins, enzymes, receptors, membrane glycoproteins and nucleic acids. Tissues exposed to oxidants thought to be involved in the I/R-induced destruction have similar structural and functional changes as I/R tissues. ROS have also been implicated in the pathogenesis of post-reperfusion myocardial "stunning", contractile dysfunction and Ca^{2+} transport (Sekili et al., 1993). Finally, oxidant production inhibitors or ROS scavengers attenuate I/R injury (Granger & Korthuis, 1995).

The natural defence to ROS in the body involves the enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (see Diagram 2), in combination with a number of non-enzymatic antioxidants such as vitamins C and E and glutathione (GSH). SOD converts the superoxide anion into hydrogen peroxide and CAT and glutathione peroxidase convert hydrogen peroxide into water and oxygen. Under hypoxic conditions, the levels of these enzymes decrease (Werns et al., 1986). It is postulated that a reduction in ROS scavengers increases the level of lipid peroxidation by ROS. The supplementation of perfusion media with CAT and SOD increases the recovery of cardiac tissue from reperfusion damage. For example, the use of a ROS scavenger such as human SOD just before the onset of reperfusion-induced damage has been shown to protect against endothelial dysfunction (Tsao & Lefer, 1990).

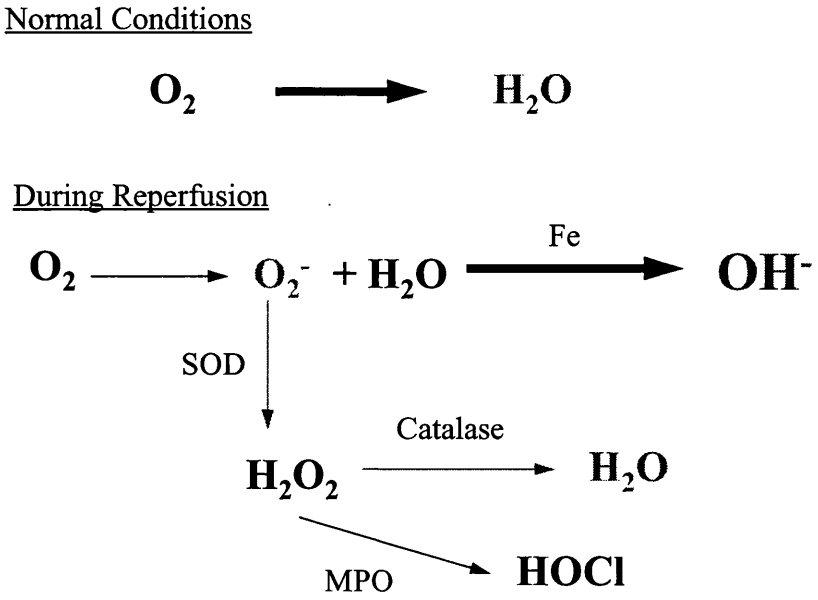


Diagram 2: A diagramatic representation of the metabolism of O_2 under normal conditions and during reperfusion.

GSH has two methods of ROS removal; firstly it can scavenge oxygen and nitrogen radicals and secondly, it donates electrons to lipoperoxide and hydrogen peroxide, effectively neutralising their effects (Leichtweis & Ji, 2001). In this study, hearts depleted of GSH and exposed to I/R were shown to display a reduction in cardiac contractility, left ventricle pressure, and left ventricle work compared with control tissues. This suggests that GSH homeostasis is important in the protection of intact hearts from reperfusion injury.

As stated above, the antioxidants vitamin C and E may be beneficial in the protection from I/R injury, for example an analogue of vitamin E such as Trolox can increase functional recovery but not reduce infarct size (Klein et al., 1991). Axford-Gately & Wilson, (1993) reported a reduced infarct size after I/R in rabbits treated with dietary supplementation of vitamin E. Vitamin E analogue supplementation may aid the prevention of reperfusion-induced arrhythmias (Walker et al., 1998). Vitamin C can decrease membrane lipid peroxidation in response to I/R induced by cardiopulmonary bypass (Dingchao et al., 1994). Moreover, hypoxic preconditioning increases functional recovery from I/R and

decreases cellular necrosis after global ischaemia via a mechanism which may be linked to the preservation of vitamin C levels (Engelman et al., 1995).

Two of the most characterised sources of ROS in I/R injury are the enzymes xanthine oxidase and neutrophil NADPH oxidase. Both enzymes are responsible for the production of superoxide and H_2O_2. In a study by Terada et al. (1992), inhibition of xanthine oxidase produced a reduction of post-ischaemic tissue injury, confirming the involvement of xanthine oxidase induced oxidants in I/R. A major cause of injury are the myeloperoxidase (MPO) and NADPH oxidase products released during the neutrophil respiratory burst. The cytotoxic effect of MPO comes from its involvement in the formation of hypochlorous acid (HOCl) from hydrogen peroxide (Harrison & Schultz, 1976) (see Diagram 2). HOCl can have detrimental effects on a number of cellular components due to its ability to oxidise or chlorinate primary amines.

It has been suggested superoxide may interact with substances in extracellular fluid to produce a leukocyte chemoattractant (Virmani et al., 1990). A study by Petrone et al., (1980) has illustrated the ability of plasma treated with an oxidant-generating system to induce chemotaxis of leukocytes; this process is inhibited by removal of superoxide. It is thought that oxidant formation during reperfusion may be involved in the expression of adhesion molecules on both endothelium and leukocytes, which may encourage the adhesion of leukocytes in post-ischaemic tissue. Neutrophil exposure to H_2O_2 produces an increase in CD11/CD18; inhibition of this process reduces the accumulation of neutrophils in the affected tissue (Suzuki et al., 1991). In cardiac myocytes, post-ischaemic cardiac lymph induces expression of ICAM-1 (Hansen, 1995). Inhibition of ICAM-1 has been shown to prevent neutrophil adhesion to peroxide-stimulated endothelial cell monolayers (Lo et al., 1993). Therefore, ROS are responsible for a number of activities in the pathophysiology of I/R-induced damage. The resultant mechanical effects of ROS include the induction of vascular contraction in isolated arteries, exacerbation of coronary endothelial dysfunction in the post-ischaemic heart and a reduction in left ventricular mechanical performance (Hansen, 1995).

The rate of production of ROS is greatly increased in the presence of the metal ions such as Fe^{3+}. In a study by Ambrosio et al., (1987), the administration of the iron chelator desferoxamine (DFO) at the time of reperfusion significantly increased recovery of myocardial function, restored normal intracellular pH levels, and gave better protection of mitochondria. Similarly Bernier et al., (1986), demonstrated a reduction in reperfusion-induced VF in hearts treated with DFO.

1.2.2. The role of leukocytes in post-ischaemic injury.

There have been a number of studies indicating the role of leukocytes in the pathogenesis of I/R injury. Firstly, granulocytes have been shown to accumulate in tissue exposed to I/R. Secondly, depletion of neutrophils significantly reduces the cellular dysfunction involved in I/R injury. Finally, inhibitors of neutrophil activation and leukocyte-endothelial cell adhesion can reduce I/R tissue injury (Hansen, 1995). Neutrophils release the proteolytic enzyme elastase and can promote activation of metalloproteinases such as collagenase and gelatinase (Weiss, 1989). Elastase appears to be the most significant effector in neutrophil-mediated damage. Due to its cationic nature it can damage tissue by altering target cell charge, or by enhancing binding to cell membranes or extracellular matrix components (Henson & Johnson, 1987). The direct effect of elastase involves the hydrolysis of proteins in the extracellular matrix, for example elastin, fibronectin, and collagen types III & IV. Furthermore, neutrophil depletion or antibody-mediated removal of neutrophils has been demonstrated to reduce lipid peroxidation, cellular dysfunction and necrosis (Romson et al., 1983, Simpson et al., 1990).

Activated neutrophils are also responsible for the release of other pro-inflammatory mediators. They can release phospholipase A_2 (PLA_2) into extracellular fluid (Lanni & Becker, 1983), thereby enhancing the production of eicosanoids and PAF by other cells (Feinmark & Cannon, 1986). The implications of these effects include a chemoattractant effect of leukotriene B_4 (LTB_4) (Linborn et al., 1982) & PAF, coronary vasoconstriction (LTC_4, D_4, E_4,

TxA_2 and PAF) and depression of left ventricular function (Lucchesi & Mullane, 1986).

The extent of neutrophil accumulation in the heart is proportional to infarct size (Smith et al., 1988). Interestingly, after short periods of ischaemia there is no associated neutrophil accumulation during reperfusion (Lo et al., 1988). However, accumulation of neutrophils in myocardial capillaries after I/R is responsible for the "no-reflow" phenomenon (Jacob et al., 1980). This effect occurs as a result of excessive endothelial cell swelling, producing large endothelial cells and protrusions which interact with neutrophils, red blood cells and platelets to block capillaries (Engler et al., 1983). This phenomenon contributes to the production of infarcted tissue, as there is poor perfusion of surrounding tissue resulting in cell death and the continuation of ischaemia. The "no-flow" phenomenon caused by neutrophils can be prevented by the administration of thrombolytic agents, and this therapeutic approach has resulted in a reduction in infarct size. For example, streptokinase and tissue-type plasminogen activator can be used to dissolve thrombi and reduce the no-flow response whilst producing vasodilation of coronary arteries (Darius et al., 1986)
The expression of adhesion proteins on the surface of leukocytes (CD11/CD18, L-selectin) and endothelial cells (P- and E- selectin) is important for the accumulation of neutrophils within an ischaemic area. CD11/CD18 expression can be increased by a number of inflammatory mediators such as LTB_4 and PAF (Harlan & Liu, 1992). Various studies using antibodies against CD11, CD18, P- and L-selectin have indicated a role for these adhesion molecules in the accumulation of neutrophils at the site of ischaemic injury (Granger & Korthuis, 1995, Lucchesi & Mullane, 1986). Furthermore, I/R-induced necrosis is attenuated when pre-treated with antibodies against CD11/CD18 (Ma et al., 1991). Finally, inhibition of P-selectin protects the ischaemic myocardium by reducing the infarct size and preserving NO production by the endothelium (Weyrich, et al., 1993).

1.2.3. Calcium-mediated injury

Increased intracellular Ca^{2+} can activate a number of signalling pathways due to its effects on various phospholipases, endonucleases and protein kinases. The level of intracellular Ca^{2+} can influence whether a cell lives or dies by its effect on DNA repair (Trump & Berezesky, 1995). There is further evidence that cellular calcium overload and oxidative stress are the two main factors involved in apoptosis or necrosis of cells exposed to I/R. Both ischaemia and reperfusion are associated with an increase in Ca^{2+} content in the damaged myocardium (Tani, 1990). This increase in Ca^{2+} content is associated with a reduction in mechanical function of the heart (Tani & Neely, 1988), and may be due to a primary event in myocardial cell injury. The increase in cytosolic free Ca^{2+} may involve disruption of the transport of Ca^{2+} across the sarcolemmal membrane (Crake & Poole-Wilson, 1986). Furthermore, the associated reduction in membrane integrity and increased permeability are also essential in allowing the diffusion of Ca^{2+} into the cells of the damaged myocardium. The increase in Ca^{2+} levels may be responsible for the induction of various arrhythmias in the ischaemic-reperfused myocardium (Corr & Witkowski, 1983). This is demonstrated by the reduction in ventricular fibrillation post I/R in greyhounds treated with nifedipine prior to the onset of ischemia (Coker & Parratt, 1983). However, Ca^{2+} antagonists used during or at the start of ischaemia do not aid recovery from I/R (Watts et al., 1980), but when administered prior to or at the start of ischaemia the extent of Ca^{2+} accumulation is reduced (Nayler et al., 1980). This suggests that L-type Ca^{2+} channels may be responsible for Ca^{2+} accumulation during ischaemia but are not effective or are inactive during reperfusion. It is also possible that there may be further factors involved in the protective response of Ca^{2+} channel antagonists. For example, Ca^{2+} antagonists may prevent the depletion of energy stores and protect the oxidation of mitochondria during I/R.

Ca^{2+} antagonists may influence Ca^{2+} accumulation by preventing a reduction in Na^+/K^+ ATPase activity (Tani, 1990). A reduction in sarcolemmal Na^+/K^+ ATPase activity in response to I/R produces an elevation of intracellular Na^+ (Van Emous et al., 1998) (see Diagram 3 (i)). The resultant decrease in pH

associated with I/R also induces an increase in intracellular Na^+ via Na^+/H^+ exchange which in turn results in Ca^{2+} loading via Na^+/Ca^{2+} exchange (see Diagram 3 (ii)). Therefore, this leads to an elevated $[Ca^{2+}]_i$ and potential myocardial damage (Allen & Xiao, 2000).

Intracellular Na^+ levels are decreased upon reperfusion via an increase in Na^+/K^+-ATPase activity despite continuing Na^+ influx during reperfusion via the Na^+/H^+ exchanger (Van Emous et al., 1998). Sarcolemmal Ca^{2+} pumps are responsible for Ca^{2+} homeostasis. During ischaemia, the activity of the sarcolemmal Ca^{2+}ATPase is reduced in membrane vesicles (Tani, 1990), producing an increase in intracellular Ca^{2+}, which is accentuated upon reperfusion of the tissue.

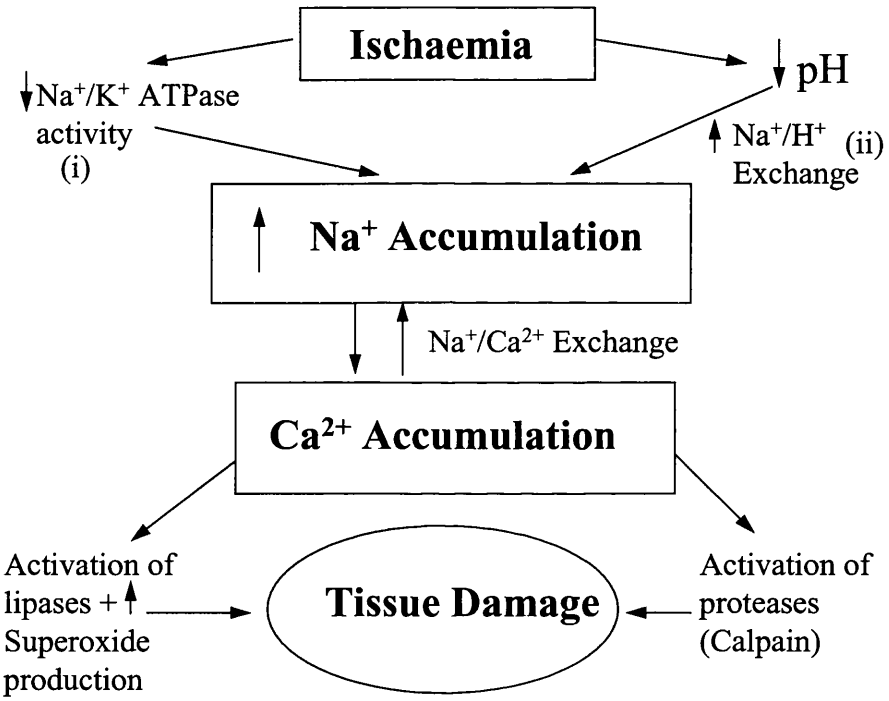


Diagram 3: The mechanisms involved in the production of Ca^{2+}-mediated tissue damage.

The balance of mediators released during I/R may have an effect on the maintenance of intracellular Ca^{2+}. Activation of α-receptors can increase Ca^{2+} influx, as demonstrated by the use of adrenoceptor antagonists (Nayler et al., 1985). A number of studies have implicated the involvement of ROS in Ca^{2+} transport in cardiomyocytes, primarily via the Na^+/Ca^{2+} exchange (Kaneko et al.,

1994). In a study by Green & Paller (1994), excess Ca^{2+} content in cells was associated with increased superoxide production via a calmodulin-dependent conversion of xanthine dehydrogenase to xanthine oxidase during I/R.

1.2.4. The role of the endothelium in I/R

During ischaemia there is a marked reduction in energy metabolism that has a profound effect on tissue injury and necrosis under conditions of ischaemia, which is accelerated upon reperfusion. During this phase, endothelial cells produce a number of mediators involved in the inflammatory response to ischaemia, including ROS (superoxide) and lipid mediators (PAF). In contrast, the endothelium is also responsible for the production and release of several factors that can reduce neutrophil attachment such as adenosine and prostacyclin (PGI_2) (Forman et al., 1990). PAF antagonists have been demonstrated to reduce microvascular leakage produced by PAF in the coronary circulation (Stahl & Lefer, 1987), and thereby prevent fluid leakage (Ma et al., 1991).

NO is an important mediator of I/R injury as indicated by the exacerbated injury seen after nitric oxide synthase (NOS) inhibition, and the increased recovery from I/R after treatment with NO donors (Bolli, 2001). As yet there is no clear explanation for the protective role of NO, but a number of hypothesised effects including antagonism of β-adrenergic stimulation and reduced contractility (Balligand et al., 1993), and opening of K_{ATP} channels (Shinbo & Iijima, 1997) have been suggested. The use of NO donors has been shown to bring about a marked increase in the preservation of endothelial function after I/R (Siegfried et al., 1992). There are disputing reports about the involvement of NO in the control of adhesion molecule expression. NO inhibition results in an increased leukocyte adherence (Kubes et al., 1991). There is also evidence of a reduced adhesion response to various inflammatory mediators in the presence of NO or NO donors (Kurose et al., 1994, Lefer et al., 1993) and neutrophil accumulation (Lefer et al., 1993). Furthermore, a study by Matheis et al., (1992), indicates that NOS inhibition prevents both the contractile dysfunction and lipid peroxidation normally associated with I/R. The damaging effects of NO are thought to be

caused by the production of the peroxynitrite anion from the combination of superoxide and NO (Beckman et al., 1990).

Prostanoids also appear to be important in the pathogenesis of I/R. In the ischemic myocardium there is a fine balance between PGI_2 and TxB_2 release, with significant increases in TxB_2 being related to increases in arrhythmia formation (Coker et al., 1981). In addition, a study by Karmazyn (1986) suggests that reperfusion is a stimulus for prostaglandin release in the rat heart, and that this may be related to the failure of ventricular recovery following I/R. In contrast, a reduction in PGI_2 production has been observed after I/R, and supplementation of PGI_2 increases the preservation of myocardial tissue from necrosis. The use of a thromboxane antagonist reduces I/R-induced arrhythmia formation, suggesting that TxA_2 may be arrhythmogenic (Coker & Parratt, 1985). The major actions of the prostanoids – namely, coronary vasoconstriction and platelet aggregation are induced by TxA_2, which will further decrease blood flow to the ischaemic area. Under normal physiological conditions, PGI_2 would act to counteract these effects, but this response is disrupted by endothelial dysfunction in ischaemic-reperfused tissue.

Endothelial cells are also responsible for the metabolism of adenine molecules and the circulating level of adenosine in the coronary circulation. Adenosine is an important mediator involved in vasodilation and coronary autoregulation. It also has the ability to disaggregate platelets and prevent the release of the constrictor TxA_2. In addition, adenosine can prevent the activation of neutrophils and the damage associated with neutrophil accumulation. This is demonstrated in a canine model of I/R, where perfusion with adenosine prevents the accumulation of neutrophils, and thus brings about a reduction in infarct size (Olafsson et al., 1987). Furthermore, adenosine and its analogues can reduce superoxide generation by neutrophils and inhibit their adhesion to endothelial cells (Cronstein et al., 1986).

I/R-induced endothelial damage may encourage procoagulant pathways due to disruption of the control mechanisms involved in coagulation. After ischaemic insult, synthesis of procoagulant enzyme tissue factors such as PAF and TxA_2,

are upregulated in the endothelium. The balance of these effects after I/R injury appears to favour thrombosis (Virmani et al., 1990).

There is speculation as to the role of heat shock proteins in the ischaemic heart. Heat shock proteins (HSPs) are a family of proteins with varying molecular weight that are expressed under conditions of stress and have been shown to have a protective effect in cells under attack. There are a number of important family members, such as HSP 70 and HSP 32 (more commonly known as haemoxygenase and discussed in length from 1.4 onwards). As regards cardio-protection, HSPs are expressed after ischaemic insult, hypoxia, haemodynamic overload and myocardial stretch (Black & Lucchesi, 1993). The extent of the protection available has been demonstrated in whole body experiments involving heat-stressed hearts exposed to ischaemia, where post-ischaemic left ventricular functional recovery is increased and enzyme loss is decreased (Yellon et al., 1992). There appears to be a direct correlation between HSP expression and protection against ischaemic damage (Yellon et al., 1992). It is possible that this may be related to an effect on neutrophil activity. Heat-induced expression of HSPs inhibits neutrophil NADPH oxidase activity, which may contribute to a reduction in superoxide production and the accompanying tissue damage (Maridonnaeu et al., 1988).

1.3. The role of pre-conditioning in the prevention of I/R-mediated damage

Pre-conditioning is a phenomenon that involves the induction of short periods of ischaemic stress in the myocardium that do not produce irreversible cellular damage, but rather activate an adaptive mechanism within the heart producing a resistance to further ischaemic insult. It generally consists of two phases, a transient early phase that occurs within minutes and a later or delayed phase that takes place several hours before activation and can last for days. There are major differences between the two phases, which is demonstrated by the different time courses involved in the responses. The early phase of the preconditioning response is mediated primarily by post-translational modifications of pre-existing cellular proteins. The later phase depends upon the upregulation of particular gene expression (Williams & Benjamin, 2000). It has been demonstrated that

preconditioning prior to full ischaemic insult decreases the extent of the myocardial injury inflicted in the coronary artery, as illustrated by a reduction in infarct size (Murry et al., 1986). Furthermore, preconditioning can increase the recovery of contractile function after I/R (Murry et al., 1986). Short periods of ischaemia prior to I/R can prevent the formation of life-threatening arrhythmias during I/R (Reimer et al., 1990). This important response may provide greater protection from ischaemic insult than the accompanying reduction in infarct size. The exact mechanism responsible for the protection from I/R injury by preconditioning is as yet unknown. A major candidate for involvement in this cardio-protective response appears to be adenosine. Several pieces of experimental evidence support this assertion: firstly, inhibition of adenosine receptors abolishes the protective effect of preconditioning in the post-ischaemic heart (Liu et al., 1991); secondly, the use of an adenosine A_1 agonist mimics the effects observed after preconditioning in the ischaemic heart (Downey et al., 1993); thirdly, adenosine has been suggested to protect the heart from ischaemia by increasing myocardial energy production from an increase in glucose transport induced by preconditioning. Another mediator thought to be involved in the protective mechanism of preconditioning is NO. The inhibition of NO generation has been demonstrated to reduce the protection evolved from preconditioning and prevent the antiarrhythmic effects of preconditioning (Vegh et al., 1992).

There is mounting evidence for the involvement of K_{ATP} channels in ischaemic preconditioning. For example, the administration of K_{ATP} inhibitors has been demonstrated to reverse the protective effect of ischaemic preconditioning in the myocardium (Auchempach et al., 1992). It has been postulated that the opening of K_{ATP} channels prior to ischaemia results in the generation of ROS, which act as a trigger for the preconditioning response (Pain et al., 2000). The K_{ATP} channel appears to be a downstream effector of adenosine as demonstrated by Van Winkle et al., (1994), where blockade of the K_{ATP} channel prevented the reduction of ischaemic injury by adenosine. Specifically, mitochondrially-expressed K_{ATP} channels have been demonstrated to be important in ischaemic preconditioning. For instance, the opening of mitochondrial K_{ATP} channels appears to be important in the response to I/R, as the K^+ channel opener

diazoxide has been demonstrated to be protective due to the induced production of ROS, and of volume regulatory actions that preserve the structure function of the intermembrane space (Dos Santos et al., 2002). Furthermore, the role of mitochondrial K_{ATP} has been directly demonstrated in ischaemic preconditioning, as the use of a selective mitochondrial K_{ATP} channel antagonist abolishes the cardioprotection induced by ischaemic preconditioning (Fryer et al., 2001). The late preconditioning effect that can be induced by adenosine A_3 receptor activation involves not only iNOS and NF-κB, but also requires the opening of the mitochondrial K_{ATP} channel (Zhao & Kukreja, 2002). Finally, the mitochondiral K_{ATP} channel's involvement in the preconditioning response to ischaemia requires protein kinase C activation and translocation to the mitochondria (Wang et al., 1999).

Protein kinase C is another downstream effector thought to be involved in ischaemic-preconditioning. Activation of protein kinase C involves its relocation to the sarcolemma where it can phosphorylate a protein target to induce the response observed. However, Valhaus et al., (1996) have suggested that protein kinase C may not be involved in ischaemic preconditioning since protein kinase C blockade using staurosporin does not prevent the induction of ischaemic preconditioning. Therefore, the degree of involvement of protein kinase C in ischaemic preconditioning is as yet unclear, but it may be possible that protein kinase C is a secondary effector in ischaemic preconditioning.

1.4 The enzyme haemoxygenase-1 (HO-1)

Haemoxygenase (HO) is an important enzyme involved in the degradation of haem molecules. Free haem occurs within the cell as a result of the breakdown of haemoglobin. As reviewed by Maines (1997), HO was first characterised in 1974 and is mainly located in the smooth endoplasmic reticulum (ER). A hydrophobic region in the carboxy terminus of the protein anchors it to the ER. HO is present in three isoforms of which HO-1 is the 32kDa inducible form of the enzyme, HO-2 is the 36kDa constitutive form, and HO-3 is the 33kDa constitutive form, of which only a little is known. Under normal physiological conditions, HO-2 is the major isoform and is expressed in a 2:1 ratio to HO-1. However, in the spleen HO-1 is constitutively expressed due to the large turnover of haemoglobin as this organ is the primary site of erythrocyte degradation. HO-1 and HO-2 are products of two different genes; whereas a single 1.8kb transcript codes HO-1, HO-2 is coded by two transcripts of 1.3 and 1.7-1.9kb. HO-1 and HO-2 both act via a similar mechanism to catalyse haem, whereas HO-3 has little catalytic activity but rather acts as a haem sensing/binding protein due to the lack of a completely conserved HO signature. The HO signature is a 24-amino acid segment also known as the haem pocket. Its main function is to bind the haem molecule via a hydrophobic bond with pyrrole rings 1 and 2, resulting in the isomer specific cleavage of the α-methene carbon-bridge. It has been demonstrated that a histidine residue within the pocket of HO-1 (His132) or HO-2 (His151) is essential for enzymatic activity. The pocket recognises the side chains of the porphyrin ring rather than the metal moiety, which means that it can be competitively inhibited by other protoporphyrins such as tin protoporphyrin (SnPP) and zinc protoporphyrin (ZnPP), as opposed to the natural substrate, FePP (haem). These molecules are commercially available as inhibitors of HO, although they cannot discriminate between isoforms.

Under normal conditions the substrate haem becomes available from a freshly synthesised haem pool prior to its incorporation into various proteins, such as haemoglobin, haem-haemopexin complexes and various cytochrome P450s (Willis, 1999). Under patho-physiological conditions, ROS and reactive nitrogen

species (RNS) generated during oxidative stress damage haemoproteins and cellular membranes, resulting in the release of haem within the cell. Therefore under these conditions it is essential that haem molecules be removed to recycle the central iron molecule and reduce the production of ROS, thus preventing further cellular oxidative stress (Maines, 1997).

The induction of HO-1 is highly sensitive to a number of stimuli that increase oxidative stress or decrease glutathione levels including heat shock (Okinga et al., 1996), ischaemia (Maulik et al., 1996b), hypoxia (Maines, 1997), inflammation (Willis, 1999), cytokines (such as TNF-α and IL-1 (Terry et al., 1998), IL-6 (Rizzardini et al., 1993) and interferon-γ (Willis, 1999), haemin (Clark et al., 2000) and a number of metal ions (Co, Fe etc) (Maines, 1997). In contrast, only adrenal glucocorticoids have been identified as chemical inducers of HO-2 (Maines, 1997). The selectivity of HO-1 is also shown by the presence of various promoter elements in the HO-1 gene, such as heat shock elements (HSE-1 and –2) (Willis, 1999), AP-1 (Lavrovsky et al., 1994), NFκB (Lavrovsky et al., 1994), hypoxic responsive elements which can bind HIF-1α (hypoxia inducible factor)(Lee et al., 1997) and metal regulatory elements (MREs) (Willis, 1999).

The catalysis of haem requires NADPH-cytochrome P450 reductase as a cofactor, and utilises two molecules of oxygen. This oxidative reaction results in the release of equimolar levels of biliverdin (further converted into bilirubin by biliverdin reductase), carbon monoxide and free iron (see diagram 4). The degradation of haem is required for the re-utilisation of iron from haemoglobin, and further prevents the generation of free radicals induced by free haem (Maines, 1997). In reducing oxidative damage caused by haem, HO-1 by-products have been demonstrated to have cellular functions of their own. For example, bilirubin is a potent anti-oxidant, carbon monoxide (CO) has vasodilator properties and free iron stimulates the induction of ferritin and the catalysis of hydroxyl radical formation.

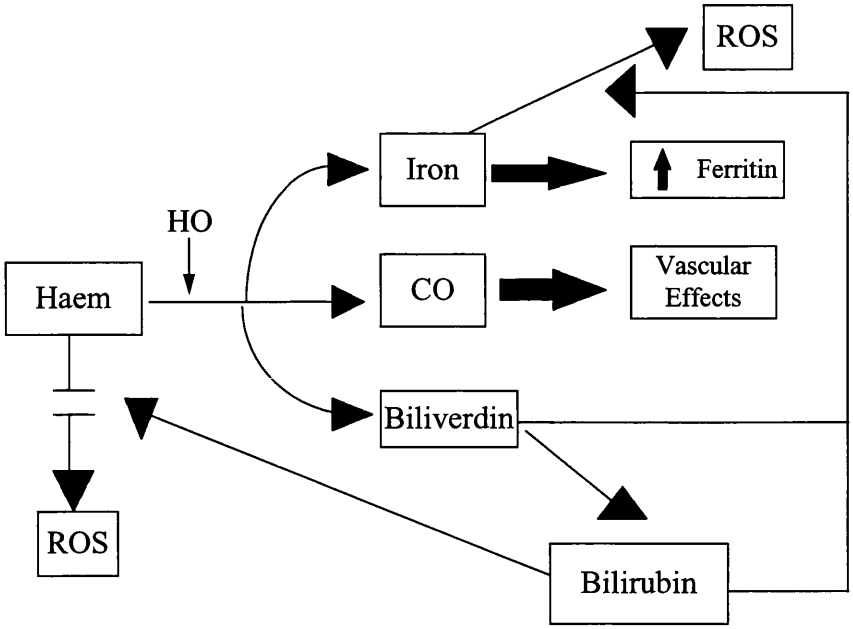


Diagram 4: HO catabolism of haem.

The function of HO-1 in physiological iron homeostasis systems has been investigated in HO-1 knockout mice. In general, the mice were smaller, thin and poorly groomed and there was also a high risk of premature fatalities. HO-1 knockout mice demonstrated a decrease in red blood cell number and size, and a decrease in serum iron accompanied by an increase in serum ferritin. There was a high proportion of kidney and liver non-haem iron accumulation. Further studies of the kidney and liver indicated increased oxidised proteins and lipid peroxidation, progressive chronic inflammatory disease and glomerulonephritis. Thus, an absence of HO-1 produced a defect in iron reutilisation, i.e. decreased delivery of tissue iron stores to the blood and decreased protection against free radical tissue damage. There is also evidence of the important role of HO-1 to prevent the induction of inflammatory disease states (Poss & Tonegawa, 1997a & b). A further, human, study of a 6-year old boy born with a HO-1 deficiency has demonstrated the potential role of HO-1 in the human body. This study reported a marked growth retardation and developmental delay associated with erythrocyte fragmentation and persistent intravascular haemolysis. Haemin – induced cell injury from the patient was increased, indicating that the presence of HO-2 did not confer protection. Therefore, this study indicates the critical

importance of HO-1 rather than HO-2 in iron metabolism and cell protection from oxidative damage (Yachie et al., 1999). In contrast, mice with an absence of the HO-2 enzyme demonstrated an increased mortality upon exposure to chronic hyperoxic exposure and increased evidence of lung oxidative injury. The results of this study indicate that abolition of HO-2 is associated with HO-1 induction and increased oxygen toxicity due to the accumulation of iron in the mouse lung (Dennery et al., 1998)

1.5 HO-1 Products (i): Bilirubin

The enzyme biliverdin reductase, which is co-expressed with HO-1, converts the breakdown product biliverdin to the bile pigment bilirubin. An interesting example of this reaction is demonstrated in the bruising of the skin. Initially the bruise is black, an indication of the release of haem, but after a couple of days this changes to a "greenish" colour after the conversion to biliverdin. Finally the fading bruise becomes a yellow colour as biliverdin is reduced to bilirubin. Bilirubin appears to act in an anti-oxidant capacity within the cell; it can inhibit lipid peroxidation (Willis, 1999), and at low O_2 tensions, it has been shown to be a more successful inhibitor of lipid peroxidation than vitamin E (Wu et al., 1991). A number of studies have indicated an anti-inflammatory role for bilirubin; for example, bilirubin increases the anti-inflammatory effect of low dose dexamethasone in a model of I/R injury in the mouse paw (Oyanagui, 1997). A further feature of both bilirubin & biliverdin may involve anti-complement effects *in vitro* (Nakagami et al., 1993). Bilirubin may have a more widespread anti-inflammatory effect, as it can inhibit the cytotoxic activity and IL-2 production from human T-lymphocytes (Haga et al., 1996).

1.6. HO Products (ii): Free Iron

Iron released from the cleavage of haem may have both protective and detrimental activity within the cell. The expected response to iron release would involve the production of reactive oxygen species as a result of the Fenton and Haber-Weiss reactions, both catalysed by free iron (Maines, 1997). A potential protective mechanism resulting from iron release may include the induction of the synthesis of ferritin, which can sequester the pro-oxidant iron (Vile et al., 1994). In HO-1 knockout cells, iron accumulation was increased and iron efflux decreased, thus providing further evidence of the importance of HO-1 in the regulation of iron mobilisation (Ferris et al., 1999). Hyperoxia-induced HO-1 expression was potentiated due to the co-release of free iron (Fogg et al., 1999). This is also demonstrated in a study by Ryter et al., (2000), where HO-1 expression was increased after hypoxia and reoxygenation, but this response was dependent upon the release of chelatable iron. Therefore, the release of iron may be an important response in terms of acting to potentiate the expression of HO-1 and the protection induced thereby.

The pro-oxidant activity of HO occurs as a result of the release of iron. In a comparison of cell lines transfected with low, medium and high expression of HO-1, cells with high HO-1 expression demonstrated a reduced viability accompanied by an increased release of iron and an increased generation of ROS. The high level of HO-1 expression appeared to perturb the cell cycle and increase ferritin expression, and so did not successfully remove all iron present. Furthermore, the increase in iron also served to inhibit HO activity (Suttner & Dennery, 1999).

1.7 HO-1 Products (iii): Carbon monoxide (CO)

The catalysis of haem by HO results in the generation of endogenous CO. CO has an important physiological role due to its ability to activate guanylate cyclase (GC), and so bring about an increase in cGMP levels (Morita et al., 1995). It also has further properties including the ability to produce vasodilation and reduce the proliferation of vascular smooth muscle (Durante & Schafer, 1998). The release

of CO by vascular cells has both autocrine and paracrine effects on both vascular smooth muscle and circulating blood cells (see Diagram 5).

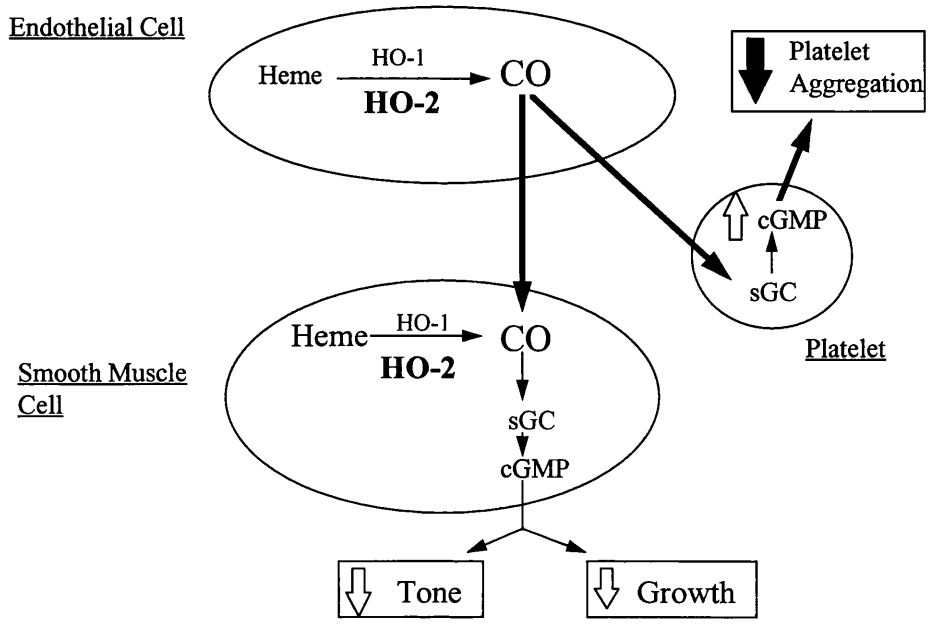


Diagram 5: The paracrine & autocrine effects of CO in the healthy blood vessels.

A number of studies have indicated a role for CO in regulating vasomotor tone. In the chronic hypoxic rat, signalling through endothelium-derived CO has been suggested as a candidate for the reduced vasoreactivity of rat aortic rings (Caudill et al., 1998). In contrast, Wang et al. (1997) have shown that CO acts independently of intact endothelium in the rat de-endotheliumised tail artery. There are other mechanisms of action by which CO may regulate vascular tone apart from a direct effect on guanyl cyclase. CO-induced vasorelaxation may be contributed to (albeit partially) by an indirect effect of prostaglandins or adrenergic effects (McFaul & McGrath, 1987). Coceani et al., (1997) proposed that the CO-induced dilation of the lamb ductus arteriosus was produced by a reduction in the formation of the constrictor endothelin-1 (ET-1). Johnson et al., (1996) reported the possible involvement of HO-derived CO as an influential factor in the pressor mechanisms controlled by the autonomic nervous system; thus, rats pre-treated with the HO inhibitor ZnPP demonstrated an increase in arterial pressure. This was also confirmed in isolated vessels, where the administration of the HO-inhibitor chromium mesoporphyrin (CrMP) caused a

sustained reduction in the internal diameter of gracillis muscle arterioles (Kozma et al., 1999).

There is strong evidence for the involvement of K_{Ca} channels in CO-induced vasodilation (Wang & Wu, 1997). In the cerebral microcirculation, CO acts as a potent vasodilator agent by acting on K_{Ca} channels (Leffler et al., 1999). Wang & Wu, (1997) discovered that CO increases the open probability of K_{Ca} channels, which may significantly affect vascular tone. CO appears to achieve this response by interacting with a particular histidine residue present in the K_{Ca} channel, thereby modulating the channel's gating mechanism. CO also appears to be very important in the regulation of cellular proliferation. For example, VSMC-derived CO has been demonstrated to modulate hypoxia-induced VSMC proliferation by suppressing the gene expression of the growth promoters ET-1 and PDGF-B (platelet derived growth factor-B) (Morita & Kourembanas, 1995). HO-1-induced CO release has also been shown to have a negative feedback effect on the proliferation of VSMCs, which is linked to a reduction in growth factor activity (Peyton et al., 2002). This has also been demonstrated in a study of neo-intimal development, where increased HO expression (induced by haemin) reduced neo-intimal formation as produced by balloon injury. This involved a NO-independent increase in cGMP, implicating the involvement of CO in the regulation of VSMC proliferation (Togane et al., 2000). However, there is some evidence that implies the involvement of CO in a pro-proliferative capacity due to prolonged exposure to CO. CO may be protective at lower doses, but after chronic exposure (for example, in long-term smokers) there may be a detrimental effect on vascular proliferation (Carraway et al., 2002).

CO has also been implicated in the protection of various organ cells from apoptosis, which is a prominent feature of a number of inflammatory diseases such as arteriosclerosis and I/R injury. Brouard et al., (2000), report that HO-1-derived CO prevents the apoptosis of endothelial cells stimulated by TNFα in a guanyl cyclase- independent manner. In addition, angiotensin II-induced hypertension increased renal cell apoptosis, which was increased by ZnPP and reduced by haemin, indicating the involvement of HO-1 in the protection of renal

cells in hypertension (Aizawa et al., 2001). This was confirmed when exposure of TNFα-treated fibroblasts to low levels of CO was shown to inhibit apoptosis via a guanyl cyclase-dependent mechanism (Petrache et al., 2000). The full extent of this protective response may involve the protection of other non-HO-1-expressing cells by CO generated by HO-1 expressing cells, such as neighbouring cells and infiltrating leukocytes (Brouard et al., 2000).

Johnson et al., (1996) observed that in rats subject to increased blood pressure (spontaneously hypertensive rats, DOCA-salt and phenylephrine-induced hypertension), addition of HO substrates (haem-L-lysinate and haem-L-arginate) induced an acute decrease in blood pressure through the production of CO. It has also been established that CO may also be responsible for the control of synaptic neurotransmission in the hypoxic lungs and airways in an *in vivo* guinea-pig model (Cardell et al., 1998). The application of exogenous CO ameliorated the degree of inflammation in the ovalbumin-induced inflammation model as measured by the amount of eosinophil accumulation (as seen in asthma), due to a decrease in the level of IL-5 production (Chapman et al., 2001). Similarly, in a model of hyperoxia-induced inflammation, exposure to exogenous CO provided protection against oxidative stress by the reduction of airway neutrophil influx (Otterbein et al., 1999). These data provide evidence of the important role CO may play in a number of situations, particularly in relation to human disease states such as atheroslerosis, hypertension, asthma and ARDS (adult respiratory distress syndrome).

1.8. Interaction of HO with NO and prostaglandins

It is interesting that HO, NO synthase and cyclo-oxygenase (COX) are all expressed in the same cell types and it is possible that there may be co-ordinated and complementary roles for these mediators. One of the main documented interactions of HO with other mediators appears to be the interaction between CO and NO. This may be due to the number of similarities between the two molecules. Both CO and NO share affinity for the haem molecule and both mediate a number of their actions via the upregulation of cGMP (Maines, 1997). However unlike NO, CO is not a free radical and cannot inflict tissue damage such as that caused by the production of the peroxynitrite radical associated with inflammation or cytotoxicity. Furthermore, HO-1 and iNOS are induced by similar stimuli such as bacterial endotoxins, cytokines and ROS (Nathan, 1992).

The interaction between CO & NO is demonstrated by the ability of each to influence the production and actions of the other. For example, it has been shown that NO and NO donors can influence the expression of HO. HO-1 mRNA or protein can be induced by the NO donors: sodium nitroprusside (SNP), S-nitro-N-acetyl penicillamine (SNAP) and 3-morpholinosyalnomine (SIN-1) in a number of cell lines (Willis, 1999). The exact mechanism of action by which NO may induce HO-1 expression is not fully known. HO-1 induction in vascular smooth muscle cells by the NO donor SIN-1 appears to be dependent on production of ROS (Durante et al., 1997). However in the same cell line treated with the NO donor SNAP-1, HO-1 induction was independent of ROS. This indicates that HO-1 induction by NO is not purely due to a change in redox state. This is further supported by the regulation of HO-1 induction at the transcriptional level by NO (Hartsfield et al., 1997). HO-1 induction in VSMCs by NO donors appeared to be independent of cGMP but dependent upon ROS (Hartsfield et al., 1997, Alcaraz et al., 2000), although it is interesting to note that in VSMCs treated with the peroxynitrite ion, HO-1 expression could not be induced (Hartsfield et al., 1997). Alternatively, recent evidence indicates that HO-1 induction may occur as a result of glutathione depletion by NO (Yee et al., 1996). NO may also regulate HO-1 activity by an indirect effect on iron metabolism, as NO is reported to regulate iron response element (IRE) binding

and ferritin synthesis (Weiss et al., 1993). A further mechanism of action may involve the release of free haem from haem proteins (Lipton et al., 1993) by NO, as HO can be induced by the presence of free haem. In conclusion, it is possible that the release of CO as initiated by NO may be a further mechanism by which NO can activate guanylyl cyclase and regulate vascular tone (Durante et al., 1997).

HO-1 can regulate the production of NO by a number of mechanisms. Firstly, increased HO-1 activity results in increased haem degradation and hence, impaired synthesis of the hemoprotein NOS. Secondly, due to the haem nature of NOS it is a substrate for HO-1 and therefore, increased HO activity results in an increased turnover of the enzyme (Maines, 1997). Furthermore, CO produced by HO can bind to NOS and effectively inactivate it (White & Marletta, 1992). Finally, iron released from the degradation of haem can inhibit the nuclear transcription of NOS (Weiss et al., 1994).

The interaction between CO and NO may have a significant influence in therapeutic situations, for example, in the ischaemic heart. NO production from L-arginine reduces the damage produced by I/R, and this reduction is accompanied by a HO-1 sensitive increase in cGMP. This suggests that although NO may be the major protective factor in this situation, CO generation is also involved in the signalling process and may potentiate the response to NO (see diagram 6) (Maulik et al., 1996).

There is also evidence of an interaction between HO and various COX products. In piglet pial arterioles, both prostanoids and NO have been shown to contribute to a CO-induced cerebrovascular dilation that is not mediated by cyclic nucleotides, but rather by activation of K_{Ca} channels (Leffler et al., 2001). Similarly, macrophages treated with zymosan (which is derived from yeast cell walls) have increased HO-1 expression, which can be negatively modulated by exogenous leukotrienes (Vicente et al., 2001). In contrast, in a macrophage cell line treated with a NO donor, HO-1 induction was potentiated by the inhibition of COX-2 (Alcaraz et al., 2001). Thus, the interaction between CO and NO or

prostanoids in both a negative and positive capacity may be important in the complex systems involved in the control of vascular tone.

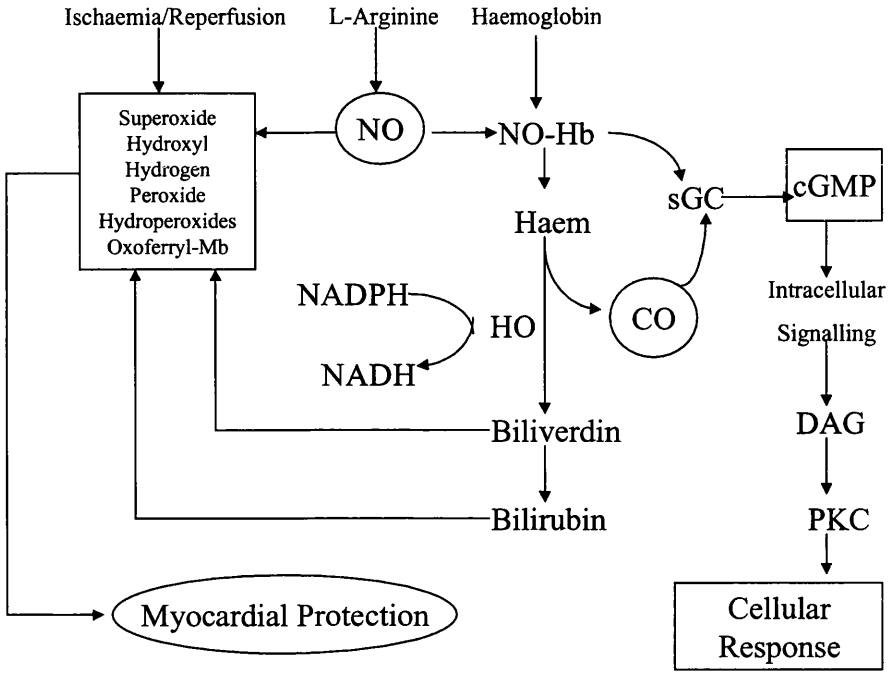


Diagram 6: A proposed interaction for CO and NO in the ischaemic myocardium (Maulik et al, 1996).
This diagram illustrates the mechanisms by which I/R could interact with NO/CO systems.

1.9 The role of HO in the cardiovascular system.

In the cardiovascular system, under normal conditions, HO-2 is the predominant form of the enzyme expressed in the endothelial and smooth muscle layers of blood vessels and in the carotid body chemoreceptors (Maines, 1997). Under conditions of stress, the HO-1 protein is upregulated in the atrioventricular node and the myocytes of the heart, as well as in the kidney and the vasculature. This has been demonstrated in an *in vivo* model of kidney I/R, which exhibits a significant increase in the expression of HO-1 in the heart and aorta (Raju & Maines, 1996). There is a high capacity in this system to generate CO, due to the high availability of the substrate haem. It is also possible that CO may contribute to cGMP generation in this system (Ewing et al., 1994).

The involvement of HO-1 in the protection of the heart and surrounding tissues has implications in a number of conditions and disease states. In inflammatory conditions such as anaphylaxis, haemin-induced HO-1 expression inhibits cardiac anaphylaxis by inhibiting the release of histamine via a cGMP-dependent mechanism which is initiated by the generation of CO (Ndisang et al., 2001). In a rat xenograft model, transplant of hearts that over-express HO-1 significantly increased the survival of the xenograft, possibly due to the anti-oxidant effect of bilirubin and the vasodilator capabilities of CO, which would increase blood flow to the new tissue (Soares et al., 1998).

HO-1 has also been implicated in the cardiovascular adaptation to hypoxic stress and in the resulting VSMC proliferation. For example, HO-1 knockout mice exposed to chronic hypoxia exhibited an increased mortality as a result of the induction of right ventricular infarction (Yet et al., 1999). Therefore, the absence of HO-1 produced a maladaptive response in cardiomyocytes to hypoxic insult, and to the resulting increase in pulmonary arterial pressure.

HO-1 may also play a role in other vascular diseases such as hypertension and atherosclerosis. In a rat model of angiotensin II-induced hypertension, HO-1 mRNA and protein was upregulated in the medial smooth muscle and adventitial cells of the aorta. It was also shown that angiotensin II had both pressor-dependent and pressor-independent effects on HO-1 regulation (Ishizaka et al., 1997). A further study of chronic angiotensin II-induced hypertension presented evidence for the expression of HO-1 in granular tissue, in inflammatory cells infiltrating the area, and in fibrous tissue deposition. The induction of HO-1 expression was found to be pressor-independent and was augmented by pressure overload (Ishizaka et al., 2000). A major feature of the progression of atherosclerosis is the induction of monocyte transmigration by mildly oxidised low-density lipoprotein (LDL). In a coculture system of human aortic endothelial cells and smooth muscle cells, LDL significantly induced the expression of HO-1. However, in haemin-treated cells the induced expression of HO-1 significantly attenuated LDL-induced chemotaxis of monocytes (Ishikawa et al., 1997).

It is postulated that, as increased ROS generation and complement activation contribute to I/R injury, the anti-oxidant and anti-complement actions of biliverdin and bilirubin could afford a protective effect (Willis, 1999). Furthermore, the release of CO may counteract the hypoxia experienced during ischaemia by relaxing coronary and aortic smooth muscles and dilating coronary arteries (Katayose et al., 1993). Maulik et al., (1996b) have described an increase in HO-1 mRNA that is dependent on the formation of ROS, upon reperfusion of the ischaemic rat myocardium. This may be due to the fact that ROS released from mitochondria during brief hypoxia have been shown to activate signalling pathways essential for the protection of cardiomyocytes against I/R injury (Foresti et al., 2001). This indicates that HO is not inducible by ischaemia itself, but rather by the oxidative stress caused during reperfusion of the ischaemic area. A number of cytokines that can induce the expression of HO have also been measured during cardiac by-pass surgery (Maulik et al., 1996b), and may be involved in the up-regulation of HO under these circumstances.

In an *in vivo* model of I/R injury, expression of HO-1 protein was not significantly upregulated until 24 hours post-insult and peaking at 48 hours (Hangaishi et al., 2000). HO-1 was primarily measured in the migrating leukocytes, and in cardiomyocytes adjacent to the infarct zone. Haemin pre-treatment also significantly reduced the infarct size compared with control 2 hours post-ischaemia (Hangaishi, et al., 2000). In contrast, in an isolated rat heart model, pre-treatment with haemin produced a significant increase in recovery from ischaemic insult due to an increase in tissue bilirubin levels that was also seen in the presence of exogenous bilirubin (Clark et al., 2000). There are a number of studies that demonstrate that HO-1 expression may provide a protective effect against I/R by an antiapoptotic effect on myocytes and endothelial cells in the affected area (Katori et al., (2002), Vulapalli et al., (2002)). Another study by Yet et al., (2001), investigated the effect of cardiac-specific expression of HO-1 on recovery from I/R injury in both *in vitro* and *in vivo* models. HO-1 over-expression increased recovery from I/R injury, indicating a role for HO-1 in myocardial homeostasis by protecting cardiomyocytes from the oxidative stress induced by I/R injury. On a cellular level, cardiomyocytes exposed to hypoxia-reoxygenation showed an increase in

HO-1 protein, which did not appear to involve ROS activation. Bilirubin production also increased after hypoxia, but not to the same extent as HO activity indicating that haem availability may be a limiting factor in the recovery of the tissue. This effect was augmented in cells treated with haemin (Foresti et al., 2001).

From the evidence discussed above, it is apparent that induction of HO-1 by I/R injury is an important mechanism involved in the protection of tissues from damage produced by ROS. Therefore, a study by Melo et al., (2002) has determined a gene therapy strategy for long-term protection of the myocardium. For example, rats subjected to intramyocardial delivery of a viral vector containing HO-1 exhibited reduced inflammation, necrosis and infarct size from ischaemic insult. This was shown to be a result of decreases in both lipid peroxidation and oxidative myocardial damage as a result of increased antiapoptotic mediator activity, accompanied by decreases in activity of pro-apoptotic mediators and pro-inflammatory cytokines. In conclusion, HO-1 expression is important in the protection of the myocardium from many different oxidative insults.

1.10 HO-1 in the kidney.

The kidney is an important organ involved in the regulation of blood pressure and blood purification. As HO-1 has been implicated in the control of blood pressure and the regulation of vascular tone, it is possible that endogenous HO-1 may have an influence on the regulation of kidney function (Da Silva et al., 2001). However, systolic blood pressure was not increased in a HO-1 knockout mouse model compared with wild-type, suggesting a role for HO-2 in blood pressure regulation (Wiesel et al., 2001).

A study on the distribution of isoform-specific HO expression in the kidney has determined that HO-2 is the predominant isoform under basal conditions. In HO-2-inhibited tissues, HO-1 expression increased and this was particularly noted in tubular and arteriolar compartments. In the cortex, HO-1 expression was observed in the proximal and distal tubules, while in the medulla HO-1 was

measured in the collecting ducts and loop of Henle (Da Silva et al., 2001). Further data have indicated a role for CO release in the control of renal haemodynamics. HO-2-derived CO has been shown to inhibit the reactivity of small renal arteries to phenylephrine (Kalab et al., 1999), and HO-1 activity is also regulated by angiotensin II in a flow-dependent manner (Ishizaka et al., 1997b). Therefore, it is possible that HO-2 released CO may influence the effect of other endogenous autacoids on the control of renovascular tone under basal conditions. A further effect of HO may involve the influence of CO on the modulation of ion transport and salt homeostasis in the thick ascending limb of the kidney (Dos Santos et al., 1998).

In a model of angiotensin II-induced renal cell apoptosis, HO-1 induction decreased the amount of cellular apoptosis (Aizawa et al., 2001). Therefore, HO-1 may exert a protective effect by controlling renal cell proliferation and apoptosis in the kidney. There is also evidence for the involvement of HO-1 in the regulation of vascular tone in the kidney through the release of CO. For example, renal arterial vessels release CO, and this can reduce the effect of constrictor agonists such as phenylephrine and vasopressin on smooth muscle by a mechanism involving activation of the K_{Ca} channel (Kaide et al., 2001). Under conditions of chronic hypoxia, HO activity is increased resulting in increased CO production and the exertion of an overall vasodilatory influence in the renal bed (O'Donaughy & Walker, 2000).

In I/R of the kidney, HO-1 expression is upregulated in response to a number of events such as reduced glutathione levels and increased ROS (Maines et al., 1993). A further study of this cellular response indicated that whilst HO-1 is upregulated in the kidney there is also an accompanying increase in HO-1 in the heart and aorta. This may be a response to stress induced by the increase in fluid volume and pressure upon reperfusion, resulting in increased expression of HO-1 in the stretch receptors of the heart and baroreceptors of the aortic arch (Raju & Maines, 1996). In the ischaemic kidney, HO-1 induction using a "spin-trap" was found to be beneficial due to the prevention of peroxidation of kidney tissue lipids. The associated preservation of the tissue structure in the cortical and

medullary regions (the areas most prone to tissue damage) was associated with high levels of HO-1 and biliverdin reductase expression (Maines et al., 1999).

1.11. Aims and Objectives

As I/R is a common pathological response in coronary heart disease, myocardial infarction and coronary bypass surgery, it is important to address the mechanisms involved and provide solutions to this problem. Therefore, the properties of HO-1 discussed earlier make it a prime candidate for further investigation as a solution to this disease.

In tissues overexpressing HO-1, it can be postulated that increased haem breakdown will result in an increase in bilirubin, CO and iron release. We hypothesised that increased HO-1 expression will facilitate increased recovery in cardiac function from I/R due to the anti-oxidant properties of bilirubin and vasodilator effects of CO, which could increase perfusate delivery to infarcted tissue. The overall aim of this study is to investigate the involvement of haemin-induced HO-1 in a rat heart model of I/R.

The Langendorff rat heart model provides a useful model for the investigation of the *in vitro* effects of HO-1 in I/R injury. The heart can be perfused using two methods; the constant-flow-perfused heart (10 ml/min) allows the maintenance of coronary flow whilst providing a model to investigate the effect of HO-1 on coronary perfusion pressure; and the constant-pressure-perfused heart (70 mmHg) maintains the perfusion pressure and allows the investigation of the effect of HO-1 on the coronary flow rates during I/R. The investigation of the effect of various treatments in response to I/R injury will be measured according to recovery from contractile dysfunction.

To investigate the effect of HO-1 expression in a model of I/R it is important to be able to induce increased expression of HO-1 in the rat heart using haemin and to measure HO-1 expression using immunoblot analysis. Further information on the mechanisms of action involved in the response to haemin under conditions of I/R, can be achieved by establishing a method to measure at least one of the

products of the HO/Haem reaction, such as bilirubin. Thus, bilirubin will be used as a biochemical marker of HO activity. The involvement of HO-1 in the response to I/R can also be determined using the HO inhibitor, SnPP.

The different methods of perfusion allow the investigation of different flow-related parameters, thus the comparison of the effect of increased HO-1 expression in both the constant-flow and constant-pressure heart models may produce important information regarding possible mechanisms involved in the response to I/R injury. Furthermore, as the degree of injury produced by I/R is proportionally related to the duration of ischaemic insult, examining the effect of haemin pre-treatment on recovery of cardiac function in the constant-flow and constant-pressure models over increased periods of ischaemia, (20, 30 and 40 min) can indicate the effectiveness of HO-1 expression in a more severe injury. The pro-oxidant effects of iron may also influence the protective effect of haemin-induced HO-1 expression, and to remove this factor the recovery from I/R can be further investigated in the presence of DFO.

Finally, the effect of haemin pre-treatment on the vascular reactivity in other vascular beds, such as the isolated kidney and the perfused mesenteric vascular bed can be investigated using tissues from previously treated rats to demonstrate the effect of HO-1 on vascular responsiveness in different tissues.

CHAPTER 2:

MATERIALS & METHODS

CHAPTER 2 : Materials and Methods

2.1 Solutions

In vitro perfusion solutions

The Krebs-Henseleit solution for heart perfusion consisted of the following (mM): potassium chloride 2.8, potassium dihydrogen phosphate 1.2, calcium chloride 1.25, magnesium sulphate 1.2, sodium chloride 118, sodium bicarbonate 25 and glucose 11.1 at a pH 7.4 gassed with 95%O_2/5%CO_2.

Heparin solution

The heparin solution for use in the isolated tissue preparations was produced from heparin sulphate (bovine) dissolved in 0.9% w/v saline at a final concentration of 1000 U/ml and stored at 4°C.

SDS-PAGE (Western blotting) solutions

Lysis buffer : 50 mM Tris HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P40, 10% glycerol, 5 mM EDTA (pH 8.0), 1 mM sodium orthovanadate, 1 mM sodium molybdate, 10 mM sodium fluoride, 40 µg/ml phenylmethylsulphonyl fluoride (PMSF), 0.7 µg/ml pepstatin A, 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 10 µg/ml soyabean trypsin inhibitor.

10X Tris/glycine: 30 M Tris HCl and 144 M glycine in 1L distilled water.

Running buffer:10% v/v 10X Tris/glycine and 10ml 10% SDS.

Transblot buffer:10% v/v 10X Tris/glycine and 20% v/v methanol.

SDS sample buffer : 0.5% w/v SDS, 50%v/v glycerol, 0.25 M Tris HCl (pH 6.8), 2-mercaptoethanol and 0.001% w/v bromophenol blue in 50ml distilled water.

1X blocking buffer : 5% w/v Marvel (powdered milk) in PBS.

4% Stacking gel : 1.33 ml acrylamide, 6.36 ml deionised water (Milli Q), 1.25 ml 1M Tris pH6.8, 0.15 ml 10% SDS.

10 % Running gel : 5 ml acrylamide, 4.35 ml deionised water (Milli Q), 5.6 ml 1M Tris pH 8.8, 0.25 ml 10% SDS.

PBS (pH 7.4): 8M NaCl, 2.9M $Na_2HPO_4.12H_2O$, 0.2M KCl, 0.2M KH_2PO_4 in deionised water (Milli Q).

2.2 The effect of haemin-induced HO-1 expression in the constant-flow-perfused rat heart model.

2.2.1. Experimental conditions for the perfusion of the constant-flow-and constant-pressure-perfused rat heart model.

This model was first described by Langendorff (1895), where the basis of the model involved the cannulation of the aorta, connecting it to a reservoir containing oxygenated perfusion fluid. This model can be used with both constant-flow and constant-pressure perfusion methods. In the constant-flow model, a flow rate of 10ml/min was selected. The main disadvantage to this system is that autoregulatory mechanisms are overridden and therefore the amount of perfusate delivered does not alter. In contrast, in the constant-pressure-perfused heart, the heart was perfused at a pressure of 70mmHg. This model was used for comparison due to its more physiological nature.

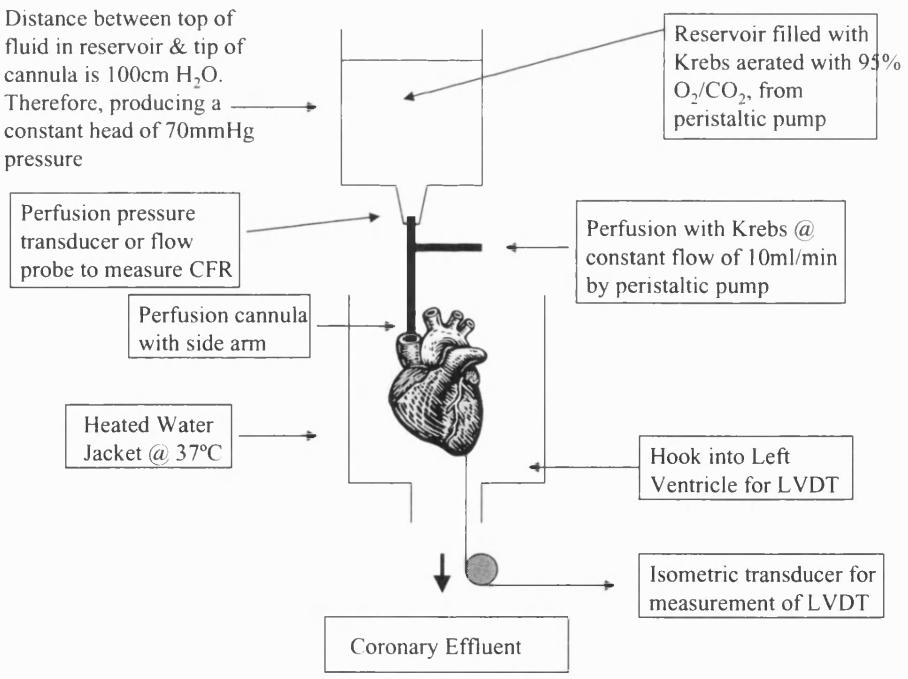


Diagram 2.2.1: The Langendorff heart perfusion system.

All animal experiments were carried out under Home Office project and personal licence specifications.

Male Wistar rats (270 - 320g – Bred in house) were anaesthetised with an injection of 100 mg/kg sodium pentobarbitone i.p. and given 500 U heparin i.v (via the rat tail vein). The heart was rapidly removed and immediately immersed in a small crucible of ice-cold Krebs to prevent spontaneous contraction. The heart was set up and perfused in the Langendorff mode via a cannula through the aortic stump at a constant flow rate of 10 ml/min using a peristaltic pump (Watson-Marlow H.R. Flow Inducer) with a Krebs solution containing 4 mM K^+ (see diagram 2.2.1). The composition of the Krebs solution used throughout the study is described in section 2.2 and was aerated with 95% O_2/5% CO_2. In the constant-flow model, the heart was perfused at 10 ml/min for a total of 30min. In the constant-pressure experiments, the heart was perfused at constant flow for 10 min before switching to a constant pressure of 70 or 130 mmHg for the remainder of the 30 min.

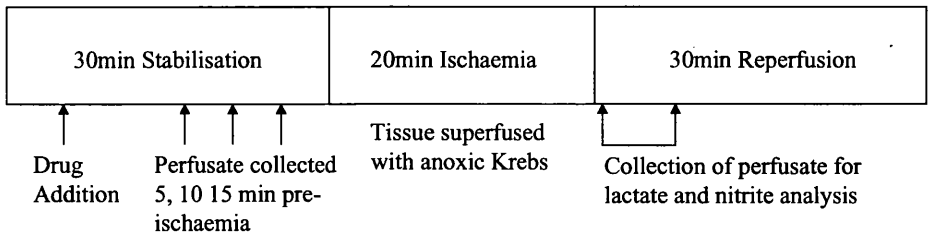
The coronary perfusion pressure (CPP) was measured using a Druck pressure transducer (Model PDCR) attached to the side arm of the aortic cannula with polyethylene tubing in the constant flow perfused heart. In the constant pressure model the coronary flow rate (CFR) was determined using a flow probe (Transonic) connected to the perfusion system before the aortic cannula. Measurements were recorded using MacLab version 3.5 on a Mackintosh Performa 5400/160. A hook attached to the left ventricle, and connected to an isometric transducer (Dynamometer UFI), was used to measure the left ventricular developed tension (LVDT). The resting tension of the heart was adjusted to 2g. The isometric transducer also triggered the measurement of heart rate (HR) via a rate meter (Lectromed). The CPP, LVDT and HR were measured using either a 4-channel chart recorder (Lectromed Multitrace) or MacLab version 3.5.

2.2.2 Investigation of the expression of HO-1 on ischaemia-reperfusion (I/R) injury in the constant-flow Langendorff heart model.

The enzyme HO-1 has been previously reported to be expressed as a result of I/R injury (Maulik et al., 1996b), and may be protective due to the release of CO and biliverdin from haem breakdown. The over-expression of HO-1 was induced in Wistar rats via an i.p. injection of haemin (dissolved in 0.1 M NaOH and adjusted to pH 7.4) at a dose of either 40 μmol/kg (Hangaishi et al., 2000) or 75 μmol/kg (Clark et al., 2000), for 18 to 24 hours respectively prior to removal of heart tissue. The HO inhibitor SnPP, when used, was administered at 40 μmol/kg i.p. 1 hour before culling the animals.

The heart was set up in the Langendorff mode and there was a stabilisation period of 30 min at either a constant-flow of 10 ml/min or a constant-pressure of 70 mmHg. The perfusate was collected for 1 min at 5, 10 and 15 min before ischaemia for the analysis of nitrite release in the constant-pressure model. The experiment followed either protocol 1 or 2 as illustrated in diagram 2.2.2, depending upon the duration of ischaemia (i.e., 20, 30 or 40 min ischaemia). During the first 5 min of reperfusion, the perfusate was collected for analysis of lactate release (and nitrite levels in the constant pressure heart model). At the end of reperfusion, the tissue was snap-frozen using liquid N_2 for the analysis of HO-1 protein levels and tissue bilirubin content.

Protocol 1:



Protocol 2:

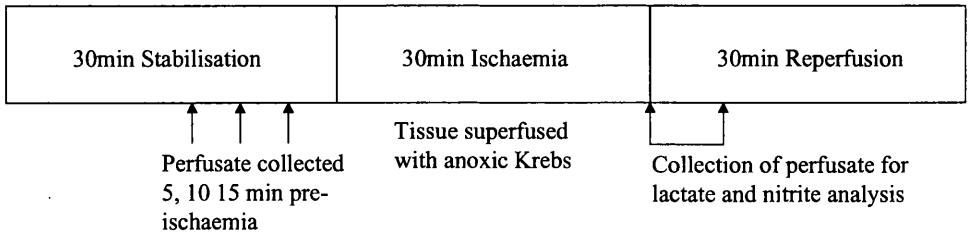


Diagram 2.2.2

Schematic representation of the protocols employed in the constant flow and constant pressure heart model.

To investigate the effect of NOS or COX inhibition in the constant-flow model, the perfusate was changed to a Krebs solution containing 100 μM L-nitro-Arginine (NOS inhibitor) or 10 μM indomethacin (COX inhibitor) after the initial stabilisation period of 10 min. To investigate the effect of iron removal in the constant-pressure model, the heart was perfused with Krebs containing 50 μM desferrioxamine (iron chelator) throughout the course of the experiment.

2.2.3. Determination of the effect of haemin-induced HO-1 expression on autoregulation mechanisms of vascular tone in the constant-pressure rat heart.

In the constant-flow model, the flow rate cannot automatically alter the amount of perfusate delivered to the heart. However in the constant-pressure model, the CFR can be altered and is controlled by autoregulation mechanisms.

Male Wistar rats were treated with 75 μmol/kg haemin or saline (24 hours) in the presence or absence of SnPP (1 hour). The hearts were removed, cannulated in the constant-flow mode and perfused at constant-flow for 10 min. The perfusion pressure was switched to a level similar to the pressure at the end of 10 min stabilisation. Therefore, control and SnPP-treated hearts were perfused at 130mmHg constant-pressure and haemin treated hearts were perfused at 100 mmHg constant-pressure. The pressure was increased or decreased to 120, 100, 80 and 60 mmHg and stabilised for 10min at each pressure. During the course of the experiment, the tissue temperature was maintained by superfusion with Krebs warmed to 37°C; LVDT, CFR and HR were measured throughout the experiment.

2.3 Immunoblot analysis (SDS-PAGE)

The separation of proteins on the basis of molecular size was obtained using the principle of SDS-PAGE as described by Laemmli (1970).

2.3.1 Protein extraction from whole tissue

Whole heart tissue samples were homogenised using 0.1 ml-lysis buffer per 100 mg tissue using a glass-Teflon homogeniser on ice. The resulting suspension was centrifuged at 14,000g for 3 min. The supernatant was transferred to a sterile 1.5 ml tube and stored at -70 ^{o}C.

2.3.2 Quantification of total protein

The protein assay of choice was based on the Bradford dye binding principal (Bradford 1976). A standard curve was obtained using a stock solution of 1 mg/ml BSA diluted with PBS (pH 7.4) to a concentration range of 1 µg/ml to 20 µg/ml. The samples were prepared by adding 10 µl sample to 0.5 ml Bradford reagent and adding 200 µl of the diluted sample or standard in duplicate to a 96 well plate. The optical density was measured using a microplate reader at a wavelength of 450 nm.

2.3.3 Sample Preparation

Protein from the supernatant of the tissue extract (10 µg) was transferred to a fresh 1.5 ml tube and resuspended in 15 µl of sample buffer. The samples were heated to 85-90 ^{o}C for 15 min and cooled on ice.

2.3.4 Gel production

This was carried out using the Protean II xi gel system (Bio-Rad). A minigel consisting of 5 % w/v stacking gel and 10 % w/v acrylamide running gel was prepared by firstly pouring the running gel so that it filled about 3/4 of the gel forming equipment. Once this had set, the stacking gel was poured and wells formed using a 9 well comb. When this had set, the gel was placed in a tank filled with running buffer. A molecular weight marker was prepared by adding 1 µl broad range molecular weight marker to 19 µl of sample buffer and loaded

onto each gel. The total volume of the sample to give 10 μg protein was loaded into each well. The gel was run at 50 V (room temperature) for approximately 30 min, or until the bromophenol blue dye front had run into the running gel. At this point, the voltage was increased to 150 V for a further 30 min or until the bromophenol blue dye front had run to the end of the gel.

2.3.5 Protein transfer

Four pieces of filter paper and a piece of nitrocellulose membrane were cut to the size of the gel. The gel, filter paper, membrane and nylon pads were assembled into a sandwich and soaked in transblot buffer to prevent shrinkage during the transblot procedure. The entire assembly was then placed inside a transblot cassette, which was immersed in a tank filled with cold transblot buffer, with the membrane adjacent to the anode. Transfer of proteins onto membrane was carried out using transblot electrophoretic equipment (Biorad) at 100 V for 1 hour. Following this, the membrane was soaked in Ponceau stain to determine successful transfer of protein onto the membrane. The Ponceau was removed by briefly washing with water followed by washing with PBS + 1% Tween. The membrane was then incubated overnight in 1X blocking buffer (as described in section 2.2) to prevent non-specific binding.

2.3.6 Immunoblot analysis for the presence of HO-1 protein

The following washes were carried out at room temperature with gentle shaking. The membrane was washed in PBS + 1% Tween for two periods of 10 min followed by three periods of 10 min in PBS alone. The membrane was incubated with mouse anti-rat HO-1 antibody (diluted 1:1000 in blocking buffer) for 2 hours at room temperature. A further washing period (as described earlier) was followed by the addition of a goat anti-mouse secondary antibody conjugated to horseradish peroxidase (diluted 1: 2000 in blocking buffer). After a further washing period, the membrane was incubated with ECL reagent for one minute. The membranes were drained and wrapped in cling film before exposure to Kodak Xomat AR5 X-ray film for between 1-4 hours.

2.4 Determination of tissue bilirubin content using a spectrophotometric method based on the measurement of azobilirubin.

The measurement of tissue bilirubin content was carried out using an adapted method from Van Roy et al., (1971) based on the conversion of bilirubin to azobilirubin and spectrophotometric analysis at 530 nm.

2.4.1 Preparation of tissue homogenates and bilirubin standards.

A sample of the frozen tissue taken at the end of each experiment was weighed and cut up into smaller pieces. The tissue was homogenised for 2 min using 0.1 ml-0.1 M NaOH per 100 mg tissue using a glass-Teflon homogeniser on ice. The resulting suspension was centrifuged at 14,000g for 3 min. The supernatant was transferred to a sterile 1.5 ml tube and used immediately.

Bilirubin standards were diluted from an initial stock solution of 1mg/ml bilirubin made up using 0.1 M NaOH. The dilutions were made using 0.1 M NaOH and were kept out of direct contact with light.

2.4.2 Preparation of the solutions required for the formation of azobilirubin

The formation of nitrous acid requires the addition of 0.15 ml of 100 mg/ml sodium nitrite to 4 ml 2M p-toluene-sulphonic acid in a sterile glass tube. The production of the diazo reagent involves the addition of 2 ml of nitrous acid to 1 ml 100 mM 4-iodoaniline. The reaction mixture was left for 1 min at room temperature. After further additions of 5 ml of distilled water, followed by 0.1 ml of 1.5 M ammonium sulphamate, the reaction mixture was mixed well. The resulting mixture was left on ice for 5 min and used immediately.

2.4.3 The formation of azobilirubin from standard or sample bilirubin.

0.1 ml of sample or standard was added to 15 ml centrifuge tubes (Falcon) and followed by 0.4 ml of an acetone-ethanol (1:1 v/v) mixture. Finally, 0.1 ml of diazo reagent was added to the reaction mixture and mixed well. The tubes were kept on ice and out of direct light for 60 min. The reaction was stopped by adding stopping reagent: 0.6 ml of 0.1 M NaCl containing 10 mg/ml ascorbic acid. At the same time, control samples were produced in the same way as reaction mixtures except that diazo reagent addition was followed immediately

by addition of the stopping reagent. n-Butyl acetate (1 ml) was then added to each tube and samples were shaken vigorously using a whirlimix (Gallenkamp) for 2 min. The upper layer was separated by centrifugation for 10 min at 6000 g and collected into cuvettes. The absorbances of samples and controls were read at 530 nm.

Bilirubin content was measured using the extinction coefficient (ε) of bilirubin in n-butyl-acetate (40 $mM^{-1}.cm^{-1}$) and the dilution factor. The results are expressed as μmol/g wet weight tissue.

2.5 Measurement of L-(+) lactate content in the coronary effluent of I/R rat hearts

The method chosen for the analysis of lactate content is based upon the method described by Hohorst (1965). The principle of the assay is based upon the following reaction:

L-(+)-lactate + NAD^+ + hydrazine –(LDH) → pyruvate hydrazone + NADH

The reaction mixture was formed as follows:

Experimental Cuvette:

450 μl 0.4 M hydrazine + 1 M glycine buffer (pH 9.5), 550 μl distilled water, 50 μl Coronary effluent sample, 50 μl β-NAD solution (40 mg/ml) and 50 U LDH (1,145 U/mg protein).

Control Cuvette:

450 μl 0.4 M hydrazine + 0.1 M glycine buffer (pH 9.5), 600 μl distilled water, 50 μl β-NAD solution (40 mg/ml) and 50 U LDH.

The lactate content of each sample was determined by the measurement of NADH production at 340 nm, after an incubation of 20 min at room temperature. The total lactate amount based on an extinction coefficient of 6.22 x 10^3 M was calculated as follows:

Vol. Of Sample X Absorbance @ 340 nm X 3.55 = Lactate μmol/min

2.6 The determination of nitrite release from the perfusate of ischaemic-reperfused rat hearts.

The method chosen for the measurement of NO involves determination of nitrite, the stable break-down product of NO. The nitrite assay selected is based upon the method of Misko et al. (1993), and detects the formation of fluorescent 1-(H)-napthotriazol from the reaction with 2,3 diaminonaphthalene.

2.6.1. Sample preparation.

The perfusate from rat hearts was collected at 5, 10 and 15 min pre-ischaemia and 1–5 min post-ischaemia. The samples were stored at –20°C for no longer than one week before use. The samples were completely thawed at room temperature before use in the nitrite assay.

2.6.2 Preparation of Standard Curve

A 1mM sodium nitrite stock solution was prepared and stored in aliquots at –20°C prior to the start of the assay. This stock solution was diluted with Krebs solution to give a final concentration range of 0.4-3 μM. Finally, a zero standard was prepared using Krebs solution alone.

2.6.3 Preparation of diaminonaphthalene (DAN)

A solution of 1 mg/ml of 2,3 diaminonaphthalene was made up in 0.62 M HCl, sonicated to dissolve and wrapped in aluminium foil. This solution was diluted in 0.62 M HCl to give a final concentration of 50 μg/ml. The solution was then stored at room temperature in the dark until use.

2.6.4 Preparation of samples and standards for nitrite assay.

For each reaction mixture, 1ml of sample or standard was added to a disposable cuvette (Elkay). 100 μl of 50 μg/ml DAN was added to each sample and mixed. The cuvettes were stored in the dark for 10 min to allow the reaction to occur. Adding 1 ml of 0.28 M NaOH stopped the reaction. Finally, the samples were read using a fluorimeter (Photon Technology International, Deltascan), (excitation 365 nm, emission 405 nm) at room temperature. The total nitrite was calculated by reference to the standard curve and dilution factor, and expressed as μg/ml.

2.7 The determination of the role of HO-1 expression in other vascular beds.

2.7.1 The rat isolated perfused mesenteric vasculature and perfused kidney.

Male Wistar rats (270-320 g) were pre-treated with 75 μmol/kg haemin or saline (24 hours) in the presence and absence of 40 μmol/kg SnPP (1 hour all given i.p). The animals were anaesthetised with an injection of 100 mg/kg sodium pentobarbitone i.p. and administered with 500 U heparin i.v. The abdomen was opened by a midline incision. For the preparation of the perfused mesentery the colic artery was tied off and the cannulation of the superior mesenteric artery was achieved via the descending aorta using a Portex cannula (size 3FG). For the preparation of the perfused kidney, the left kidney was cleared prior to cannulation of the renal artery via the descending aorta with a Portex cannula (size 3FG). The mesentery and kidney were carefully separated from the intestines and mounted vertically in a heated water jacket at 37°C. The mesentery and kidney were perfused at a constant-flow of 6 ml/min and 5 ml/min respectively by a peristaltic pump (Watson-Marlow 501) with Krebs-Henseleit solution containing 4 mM K^+ (warmed to 37°C and aerated with 95% O_2/5% CO_2). The perfusion pressure was measured using a Druck PDCR75 pressure transducer and recorded using MacLab version 3.5 on a Macintosh Performa 5400/160.

A stabilisation period of 30 min was allowed before the start of the experiments. All drugs were administered as a bolus injection of no more than 50 μl via an injection approximately 5 cm from the preparation. For the investigation of vasodilator substances ACh and histamine, the mesentery and kidney were pre-contracted using 10 μM phenylephrine (PE) dissolved in Krebs solution or Krebs solution containing 30 mM K^+ respectively, perfused for 30 min prior to the start of the experiment. For the investigation of the vasoconstrictor agents PE, endothelin-1 (ET-1) and sarafotoxin-6C (SX6C) (only recorded in kidney), each dose followed a dose cycle time of approximately 5 min and at least 10 min between each dose response curve in the above order.

Stock solutions of PE, histamine, ET-1 and noradrenaline (Krebs solution + ascorbic acid) were made up using Krebs solution at a concentration of 10 mM and stored at -20°C. Further dilutions were made using Krebs solution for use during the experiment.

2.8 Statistical analysis

All values are expressed as mean ± standard error of the mean (SEM).

Statistical differences were compared by either Student's t-test for comparison between two groups, or ANOVA (one-way) and a comparison of means using Tukey's Honestly Significant difference test when more than two groups were involved. Significance was measured as $P<0.05$.

The recovery of cardiac function after I/R was expressed as % of the original pre-ischaemic value compared with the post-ischaemic value.

2.9 Materials

Male Wistar rats (270 - 340 g) were bred in house.

Sigma supplied: acetone, acetylcholine chloride, ammonium persulphate, aprotinin, ascorbic acid, bilirubin, bovine serum albumin (BSA), bradykinin, bromophenol blue, n-butyl acetate, calcium chloride, EDTA, endothelin-1, glycerol, glycine, haemin sulphate, histamine, lactic dehydrogenase (LDH), lauryl sulphate (SDS), leupeptin, magnesium sulphate, maleic acid, 2-mercaptoethanol (2-ME), nonidet P40, noradrenaline, pepstatin A, phenylephrine, phenylmethylsulphonyfluoride (PMSF), ponceau, sodium acetate, sodium fluoride, sodium hydroxide, sodium molybdate, sodium nitrite, sodium orthovanadate, soyabean tetramethylethylenediamine (TEMED), trizma base, trypsin inhibitor (STI), and tween-20.

β-NAD (Boehringer Mannheim).

Hydrazinium sulphate (Fisons).

Tin protoporphyrin (Tocris).

Ethanol, glacial acetic acid, glucose, KCl, KH_2PO_4, methanol, NaCl, nitrocellulose membrane and sodium bicarbonate (BDH).

Acrylamide (National Diagnostics).

Bradford protein assay reagent and broad range molecular weight marker (Biorad).

Mouse anti-rat HO-1 monoclonal antibody (Stressgen).

Goat anti rat conjugate antibody (Dako).

Chemiluminescent (ECL) reagent (Amersham International).

Ammonium sulphamate, 4-iodoaniline, p-toluene-sulphonic acid (Acros)

Desferrioxamine (Ciba Labs).

CHAPTER 3:

THE ROLE OF HO-1 IN THE CONSTANT-FLOW PERFUSED RAT HEART

CHAPTER 3: The role of HO-1 in the constant flow perfused rat heart

3.1. The development of methods used throughout the investigation.

3.1.1 The determination of the effect of haemin and SnPP treatment on HO-1 expression in the rat heart

HO-1 expression can be induced by a number of stimuli such as cytokines, hypoxia, metal ions, glutathione removal and haemin. For the purpose of this study, HO-1 expression was induced using an i.p. injection of haemin. A previous study comparing the effects of 40 μmol/kg haemin (18 hours) and 75 μmol/kg haemin (24 hours) (Data not shown) on HO-1 expression in the rat heart found the higher dose of 75 μmol/kg haemin produced a larger increase in HO-1 expression. The effect of the HO inhibitor SnPP on HO-1 expression was investigated using immunoblot analysis with an anti-rat HO-1 antibody (Fig 3.1.1).

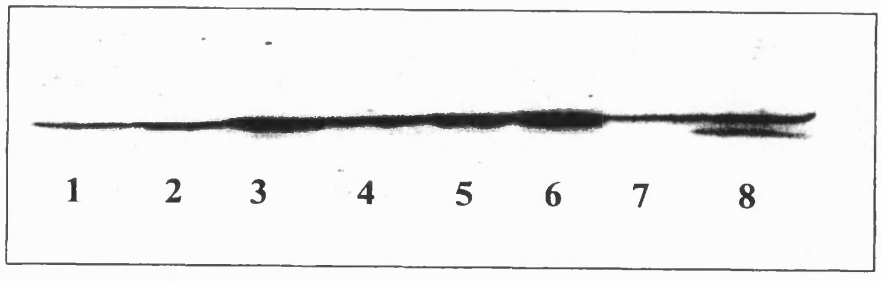


Figure 3.1.1
The effect of haemin pre-treatment on HO-1 expression in the rat heart.
Rats were treated with saline (1 & 2), 75μmol/kg haemin (24hours) (3 &4), 75 μmol/kg haemin + 40μmol/kg SnPP (5 &6) and 40μmol/kg SnPP alone (7 & 8).
Hearts were frozen using liquid nitrogen after 30min perfusion followed by 20min global ischaemia and finally 30min reperfusion.

The results clearly demonstrate that 75 μmol/kg haemin produces an increase in HO-1 expression (3 & 4) compared with control (1 & 2). However, SnPP treatment (in the presence of haemin, 5 & 6) did not affect HO-1 protein levels. It is also important to note that SnPP alone did not induce HO-1 protein expression (7 & 8). The data presented in figure 3.1.1 are representative of the increase in HO-1 expression observed throughout the study. Analysis of HO-1 protein levels was carried out after each experiment with the same result.

3.1.2 The evaluation of the method selected for the determination of tissue bilirubin levels.

The initial experiment involved the determination of a standard curve of bilirubin over a range of 0-100 μg/ml. This was achieved by following the protocol described by Van Roy et al. (1971). Bilirubin was extracted from the sample and converted into azobilirubin, whose absorbance could be measured spectrophotometrically at 530 nm. The bilirubin was dissolved in 0.1 M NaOH at an initial concentration of 1 mg/ml, and further dilutions were made to cover a range of 0-100 μg/ml.

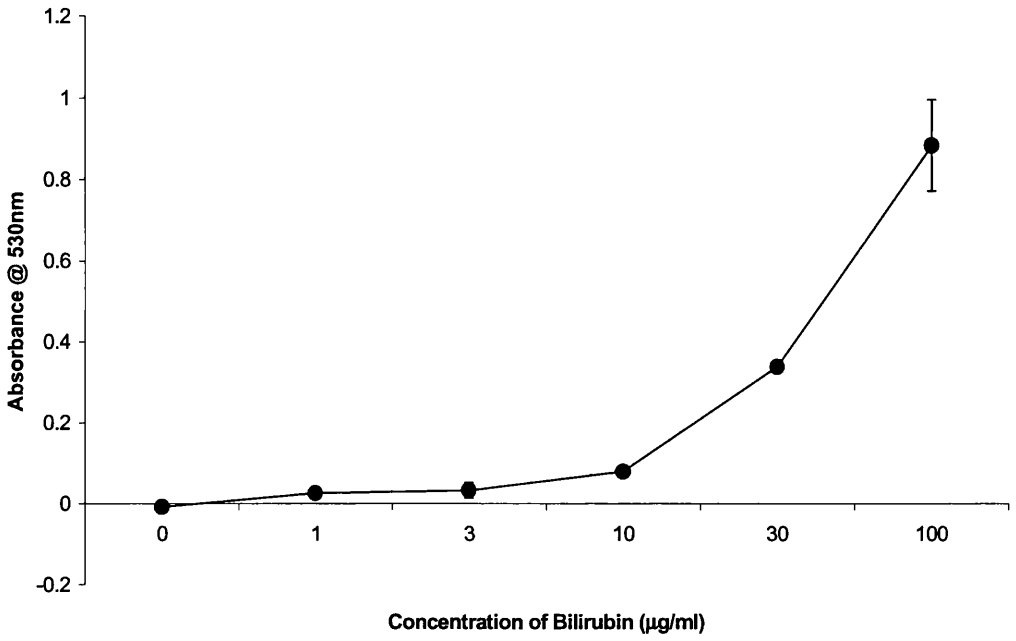


Figure 3.1.2
A standard curve for bilirubin over a range of 0-100 μg/ml.
A series of bilirubin solutions covering the range of 0-100 μg/ml were converted into azobilirubin and measured at 530 nm. Values were measured mean ± SEM after conversion to azobilirubin, where n=4. Note: some errors fall within symbol size.

This initial experiment demonstrated that the bilirubin concentration of a stock solution could be determined using the method selected. The next step was to determine if bilirubin could be measured from a homogenised tissue sample. The original data (not shown) demonstrated that bilirubin was not detected in spleen tissue, although this tissue is reported to have a high level of HO-1 expression (Maines, 1997). The following experiment was used to compare the effect of a

bilirubin stock solution dissolved either in lysis buffer and 0.1M NaOH on the detection of bilirubin levels (Figure 3.1.3).

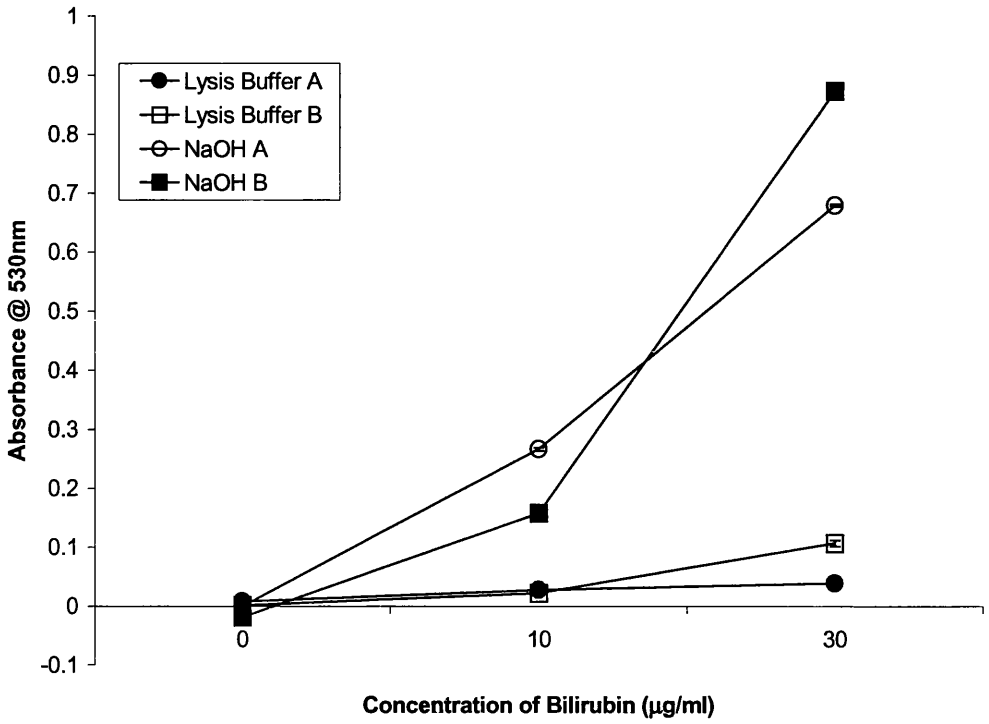


Figure 3.1.3
The comparative effect of lysis buffer or 0.1M NaOH on bilirubin measurement.
Values are expressed as mean ± SEM (errors fall within size of symbol), on two separate occasions denoted as A and B, (n=4).

The data described in figure 3.1.3 demonstrates that bilirubin may dissolve more readily in NaOH than lysis buffer or that other elements of the lysis buffer affected absorbance. Furthermore, the data is also fairly reproducible as indicated by similar results achieved on two separate occasions (A and B). Therefore, in all subsequent test, tissues were homogenised in 0.1 M NaOH.

The final experiment investigated the efficiency of the assay by measuring a known concentration of bilirubin added to a homogenised tissue sample (Figure 3.1.4). The "spike" test demonstrates the accuracy of the assay, by measuring a known amount of bilirubin extracted from a tissue sample. A bilirubin solution of 100 µg/ml bilirubin produced an absorbance of 0.382±0.05 (n=4), compared with 0.586±0.01 (n=4) in samples containing spleen and bilirubin, and 0.262±0.04 (n=4) for spleen alone (see methods pg 51). Therefore, extraction of bilirubin using n-butyl acetate was shown to exceed 85%.

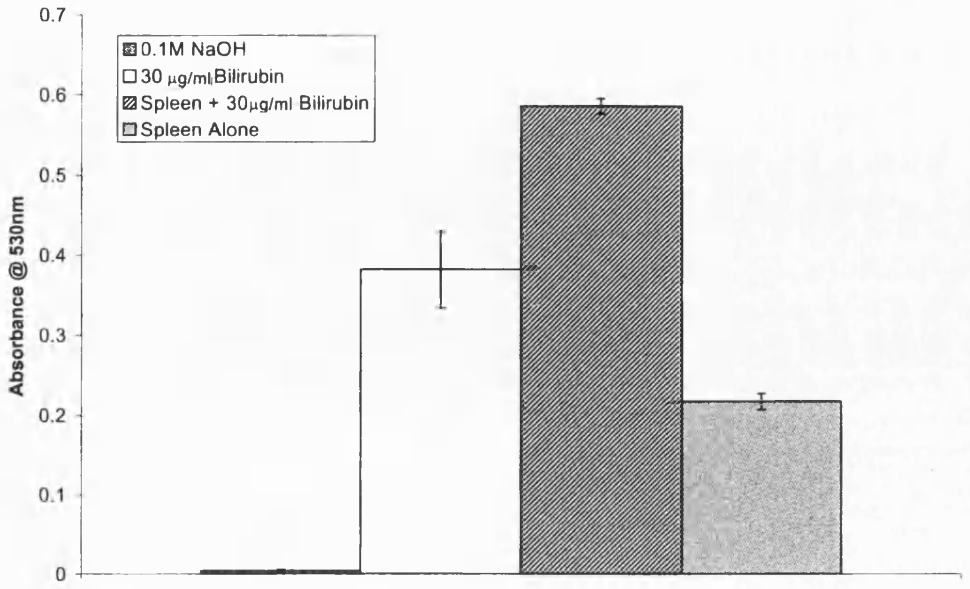


Figure 3.1.4
The accuracy of the Azobilirubin assay as measured by the extraction of a known bilirubin concentration from a tissue sample and compared with the absorbance of the tissue sample alone. Values are expressed as mean±SEM, (n=4).

3.2 The effect of haemin pre-treatment on the recovery from I/R in the constant-flow perfused rat heart.

The effect of haemin on the recovery of cardiac function after I/R was investigated to determine whether any potential increase in HO-1 activity might offer protection. It is possible that the increase in haem breakdown and the accompanying increase in HO by-products may act to "pre-condition" the tissue, and thereby improve the recovery from I/R in the constant-flow-perfused rat heart model. In addition, the effect of haemin-induced HO-1 expression was also measured in the pre-ischaemic heart.

3.2.1 A representative description of the experimental procedure and observations / measurements made during procedure.

The traces shown in Figures 3.2.1A and 3.2.1B are representative of the standard I/R (20 min ischaemia/ 30 min reperfusion) experiment under constant-flow conditions in haemin-treated and control hearts respectively. The hearts were set up as described in section 2.2; during the initial 30 min of perfusion, the CPP increased and eventually stabilized at around 100 mmHg (section A-trace). The heart rate remained relatively constant throughout initial perfusion stage even during the adjustments to basal tension (section B-trace). Finally, the developed tension remained low for about 5-10 min and basal tension was adjusted until at the 30 min stage developed tension stabilized at around 6-8g (section C-trace). During zero flow global ischaemia there were no measurements of CPP. There were some small contractions that triggered the rate-meter during the first 2-3 min of ischaemia. After 20 min ischaemia, the perfusate was re-introduced and contractions developed within the first two min. As this is a haemin-treated heart the contractions are large enough to trigger the rate-meter. Unfortunately, contractile recovery is very poor in control hearts and the rate meter was not triggered making it difficult to compare HR data. The developed tension, CPP and HR (if possible) were measured throughout the 30 min reperfusion period.

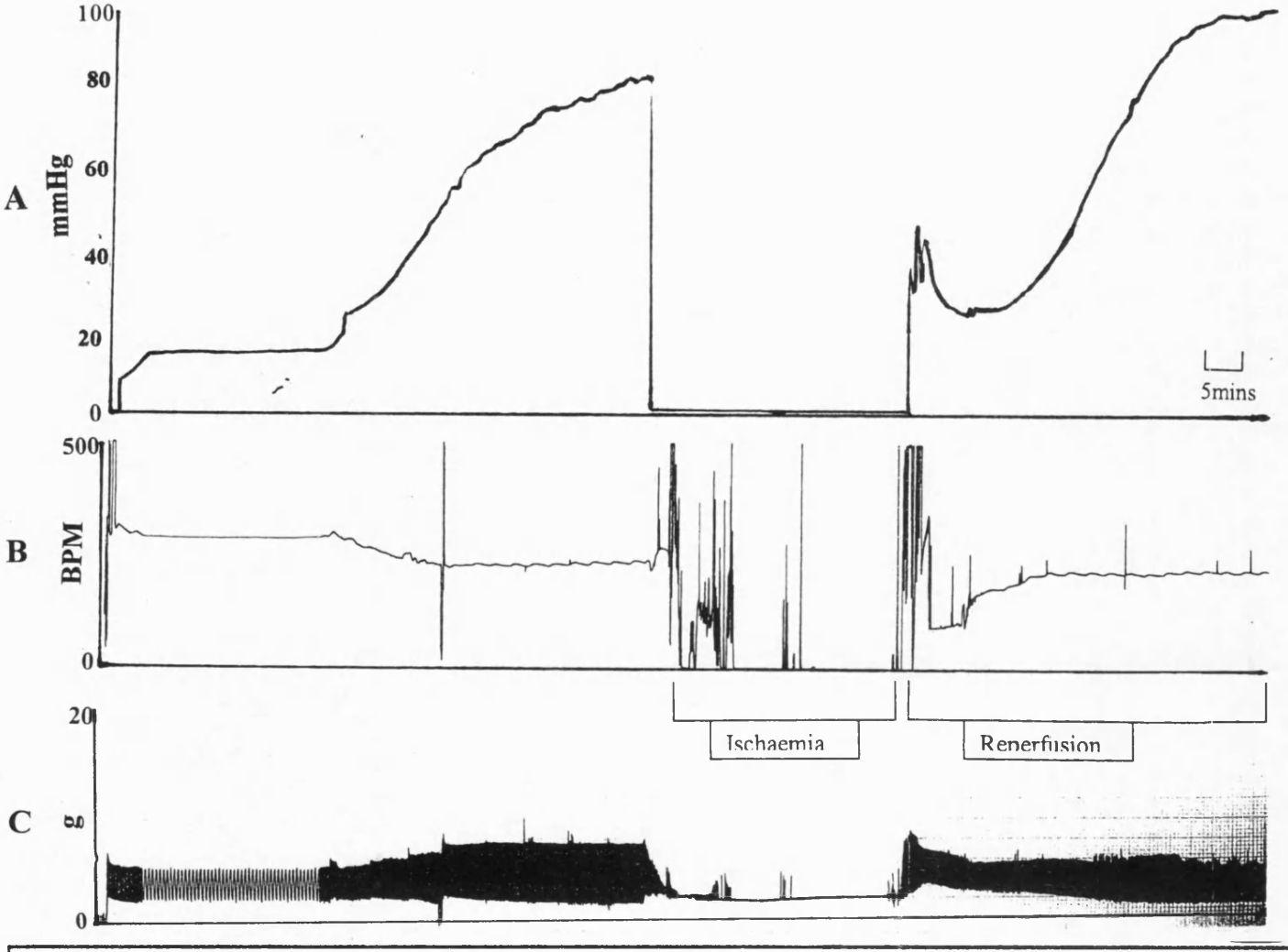


Figure 3.2.1A

This trace is representative of the experimental conditions and observations in a haemin-treated heart perfused at a constant-flow of 10ml/min. The CPP (section A), heart rate (section B) and LVDT (section C) were measured throughout the initial stabilisation period, 20min ischaemic insult and 30 min reperfusion.

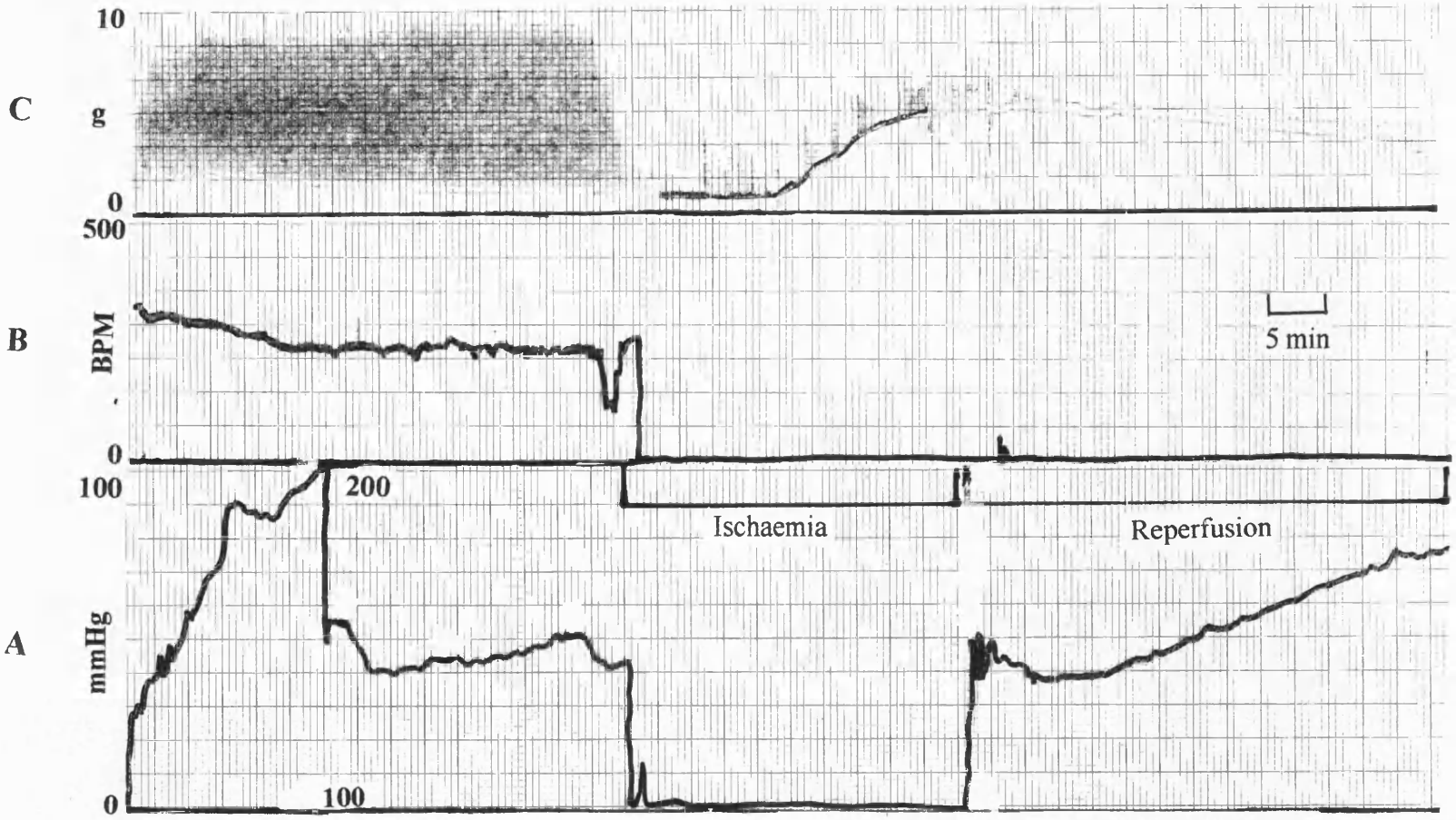


Figure 3.2.1B

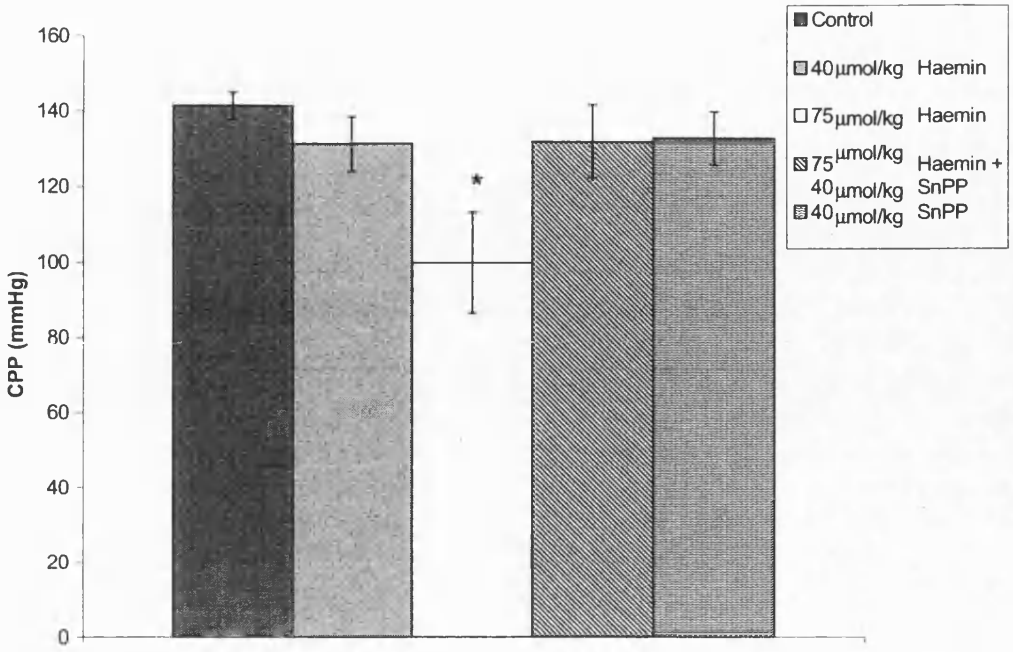
This trace is representative of the experimental conditions and observations in a control heart perfused at a constant-flow of 10ml/min. The CPP (section A), heart rate (section B) and LVDT (section C) were measured throughout the initial stabilisation period, 20min ischaemic insult and 30 min reperfusion.

3.2.2. Pre-ischaemia

After a stabilization period of 30 min, CPP in control hearts was recorded as 141.3±3.6 mmHg (n=5), compared with 131.3±7.2 mmHg and 99.8±13.5 mmHg after 40 μmol/kg and 75 μmol/kg haemin treatment respectively (figure 3.2.2A). Therefore, haemin pre-treatment reduced CPP in a dose dependent manner. 75 μmol/kg haemin, the highest dose used, significantly decreased CPP compared with control ($p<0.05$, n=5). This suggests that there is a vasodilator component involved in the response of the pre-ischaemic rat heart to haemin pre-treatment. The HO inhibitor SnPP abrogated the effect of haemin on CPP, while having no effect on its own. Therefore, these data implicate HO in the vasodilator response to haemin. Haemin pre-treatment had no significant effect on the developed tension compared to control (5.65±1g) (Fig 3.2.2B). In contrast, SnPP alone significantly increased contractility, which implicates that HO-1/HO-2 may be involved in the control of endogenous contractility of the constant-flow-perfused rat heart.

At the end of the initial 30 min perfusion period, haemin pre-treatment at either dose did not significantly affect the heart rate (270±18 BPM after 40 μmol/kg haemin compared with 284.6±18 BPM for 75 μmol/kg haemin) of the tissue compared with control (285.8±19 BPM) (Figure 3.2.3A). Interestingly, there was no variation in the response to haemin after SnPP treatment (272.5±12 BPM) but tissues exposed to SnPP treatment alone demonstrated a significant increase in HR compared with control (362.5±112 BPM, $p<0.05$, n=5). These data contrast with the results observed in figure 3.2.2B, where SnPP alone significantly increased LVDT compared with control levels. Therefore, there is strong evidence that HO has a significant influence on the contractility of the rat heart in this system. At this stage it is unclear by which mechanism (s) HO may exert such an effect. The measurement of the basal tension is an indication of potential Ca^{2+} overload. During the equilibration interval, haemin and SnPP pre-treatment did not significantly affect basal tension (Figure 3.2.3B). Therefore, these data suggest that after haemin and/or SnPP treatment, the heart is stable and viable for use in the I/R experiment.

(A) CPP



(B) LVDT

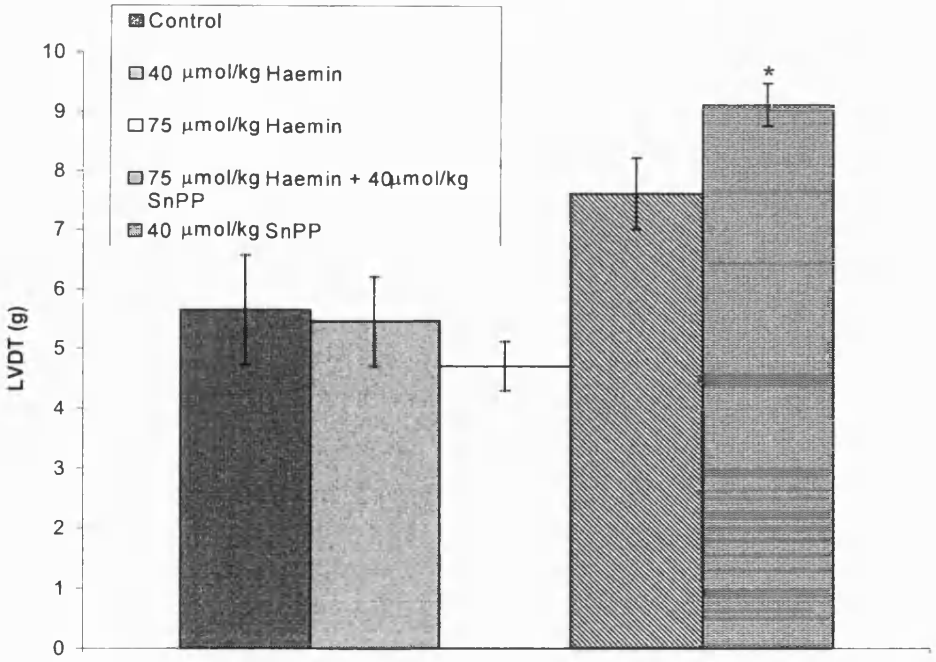
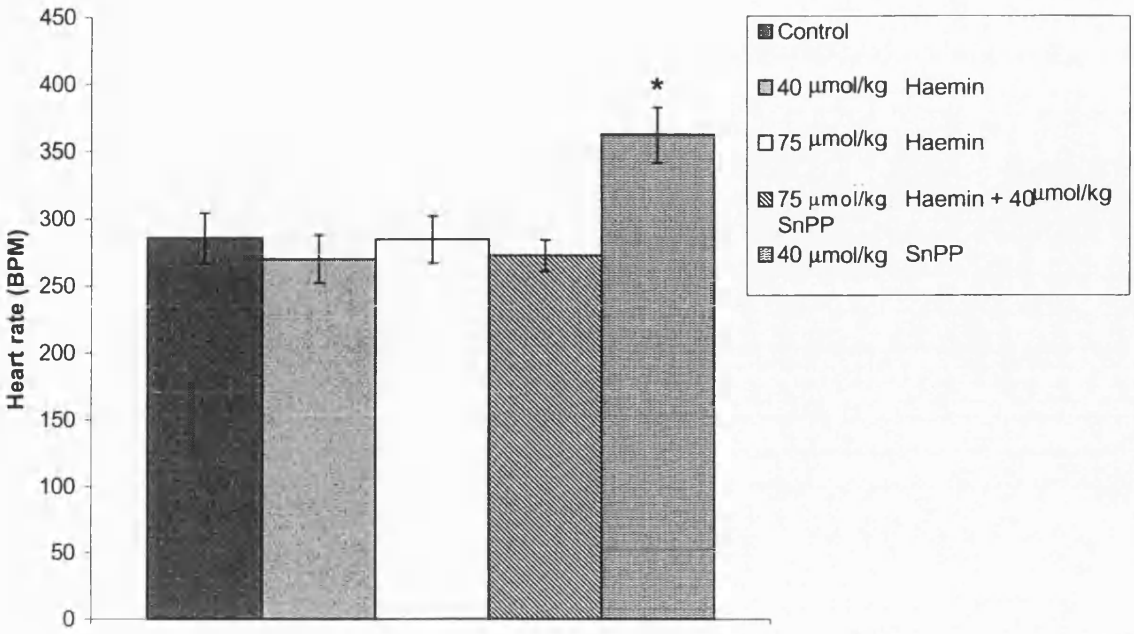


Figure 3.2.2

The effect of haemin and SnPP pre-treatment alone and in combination on CPP (A) and LVDT (B) in the pre-ischaemic rat heart. Haemin doses are as follows; 40 µmol/kg haemin (18hour) and 75 umol/kg haemin (24hour), 40 µmol/kg SnPP (1hour) or 75 µmol/kg haemin + 40 µmol/kg SnPP. Values are expressed as mean±SEM, where * is $p<0.05$ compared with control (n=5).

(A) Heart Rate



(B) Basal Tension

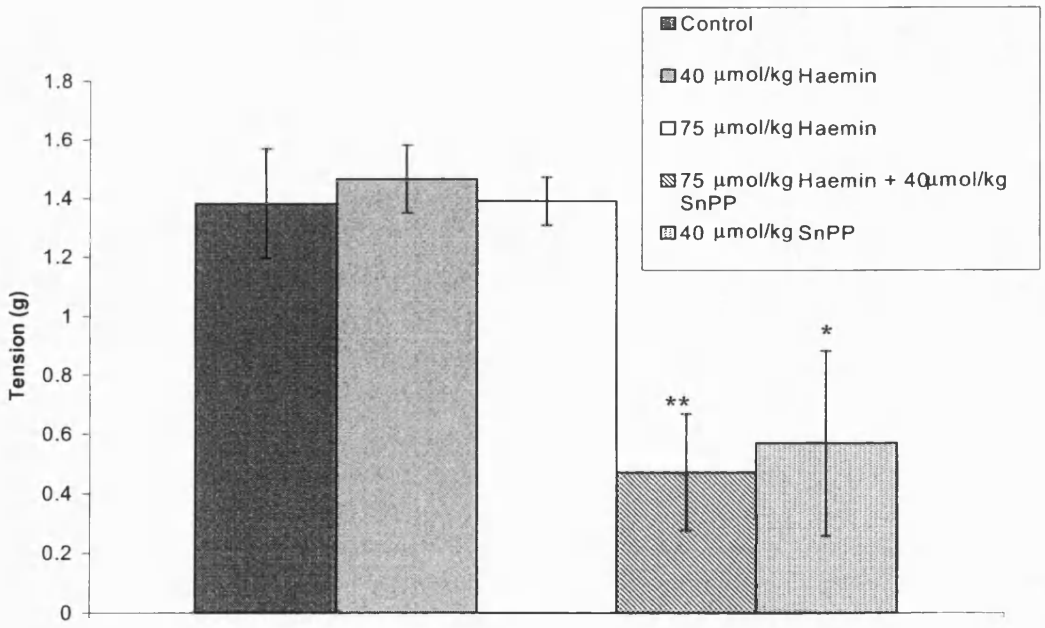


Figure 3.2.3

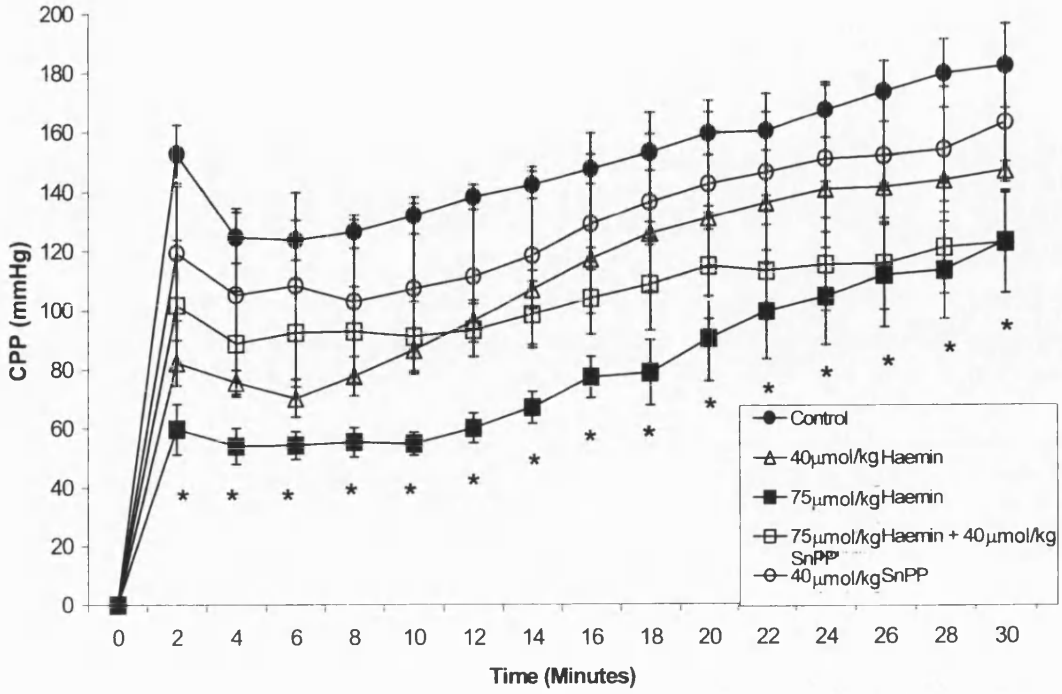
The effect of haemin and SnPP pre-treatment alone and in combination on Heart Rate (A) and basal tension (B) in the pre-ischaemic rat heart. Haemin doses are as follows; 40 µmol/kg haemin (18hours) and 75 umol/kg haemin (24hours), 40 µmol/kg SnPP (1hour) or 75 µmol/kg haemin + 40 µmol/kg SnPP. Values are expressed as mean±SEM, where * is $p<0.05$ compared with control and ** $p<0.05$ compared with 75 µmol/kg haemin (n=5).

3.2.2. The response of the rat heart to haemin pretreatment post ischaemia.

After a period of 30 min stabilization, hearts were subjected to 20 min ischaemia followed by 30 min reperfusion to investigate the effect of HO-1 induction on the recovery of cardiac function after I/R. Haemin pre-treatment produced a dose dependent reduction in CPP throughout reperfusion compared with control. This confirms the data recorded prior to ischaemia, where the higher dose of 75 μmol/kg haemin significantly decreased CPP compared with control for the entire 30 min (see figure 3.2.4A) ($p<0.05$, $n=5$). In conclusion, haemin pre-treatment significantly decreased CPP during reperfusion indicating a vasodilator response. The addition of SnPP had no significant effect. These data indicate that the vasodilator effect is not dependent upon ischaemic insult as it was demonstrated in both the pre- (Fig 3.2.2A) and post-ischaemic periods. It is possible that a HO-1-mediated reduction in CPP may not be the only mechanism involved. The CPP values of SnPP and control hearts during reperfusion did not significantly differ, indicating that endogenous HO is unlikely to be involved in the pathological response to I/R.

The recovery of cardiac contractility during reperfusion was measured as the % recovery of the contractility of the tissue (after 30 min reperfusion) compared with the pre-ischaemic value. Haemin pre-treatment increased the recovery of cardiac contractility during reperfusion in a dose-dependent manner (20 ± 6.7% after 40 μmol/kg haemin, and 45.3 ± 8.7% after 75 μmol/kg haemin) compared with control (14.3 ± 4.5%) (figure 3.2.4B). The highest dose of haemin significantly increased recovery compared with control ($p<0.05$, $n=5$). SnPP treatment did not significantly affect the recovery of contractility in either the presence or absence of haemin (27.3 ± 13.8% and 7.2 ± 3.3% respectively). In conclusion, HO-1 induction significantly increases recovery of cardiac function after I/R, but it is as yet unclear if HO-1/HO-2 is involved in the endogenous response to I/R. The effect of I/R on HR could not be determined due to the lack of data from the rate meter, which was not triggered consistently by the developed tension recorded.

(A) CPP - during reperfusion



(B) Recovery of LVDT

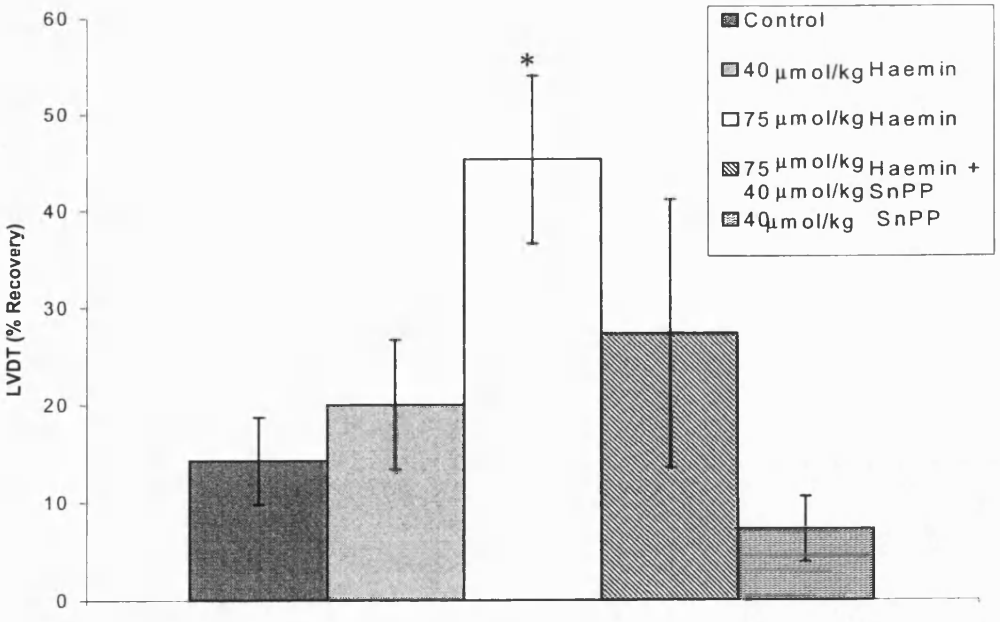
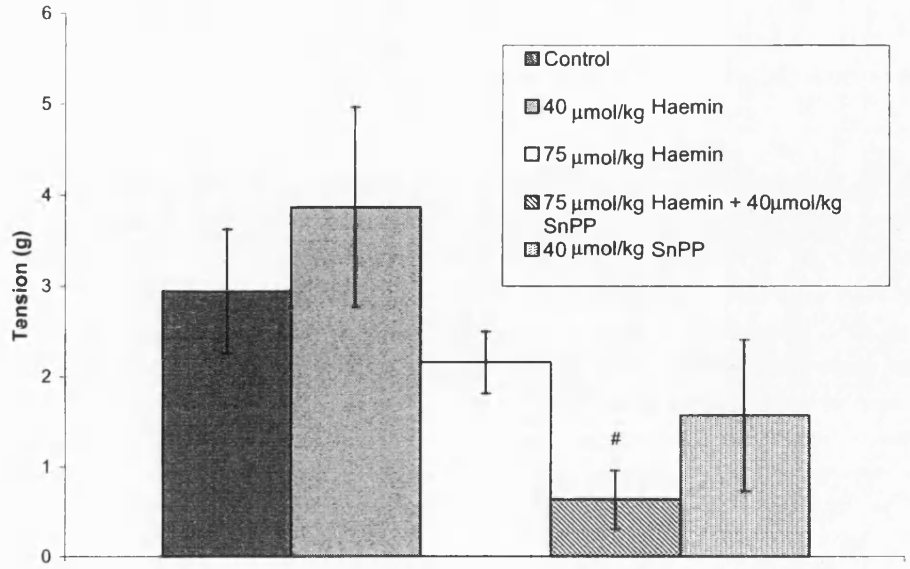


Figure 3.2.4
The effect of haemin and SnPP pre-treatment alone and in combination on CPP (A) and recovery of LVDT (B) during reperfusion (30min).
Hemin doses are as follows; 40 µmol/kg haemin (18hour) and 75 umol/kg haemin (24hour), 40 µmol/kg SnPP (1hour) or 75 µmol/kg haemin + 40 µmol/kg SnPP. Values are expressed as mean ± SEM, where * $p<0.05$ compared with control (n=5).

Treatment with SnPP in the presence of haemin significantly reduced basal tension compared with control and haemin alone. Thus, it is possible that in combination, haemin + SnPP treatment produces an additive effect. As this effect is not paralleled by the recovery of cardiac function this may be an indirect effect of haemin and SnPP treatment, as opposed to being due to HO-1 induction and consequent inhibition (Figure 3.2.5A).

At the end of reperfusion, the tissue was frozen using liquid N_2 and stored at -80°C for the measurement of tissue bilirubin levels (Figure 3.2.5B). 40 μmol/kg haemin did not have any effect on bilirubin production compared with control. In contrast, 75 μmol/kg haemin significantly increased bilirubin production (6.4 ± 0.6 μmol bilirubin/g wet weight tissue) compared with control (3.5 ± 0.6 μmol bilirubin/g wet weight tissue, where * $p<0.05$, $n=5$). The addition of 40 μmol/kg SnPP did not significantly reduce the amount of bilirubin measured after haemin pre-treatment (5.1 ± 1.1 μmol bilirubin/g wet weight tissue). SnPP alone also failed to significantly reduce tissue bilirubin levels (2.5 ± 0.9 μmol bilirubin/g wet weight tissue) compared with control. Tissue bilirubin levels are an indication of HO activity and provide a possible mechanism for the protective action of HO-1. These data show that pre-treatment with high doses of haemin significantly increased tissue bilirubin production. This is likely to be due to the increased availability of substrate, as haemin is a known inducer of HO-1 because it is the common substrate of the HO-mediated reaction producing a 1:1:1 ratio of bilirubin, CO and iron.

(A) Basal Tension – during reperfusion



(B) Tissue bilirubin production – post I/R

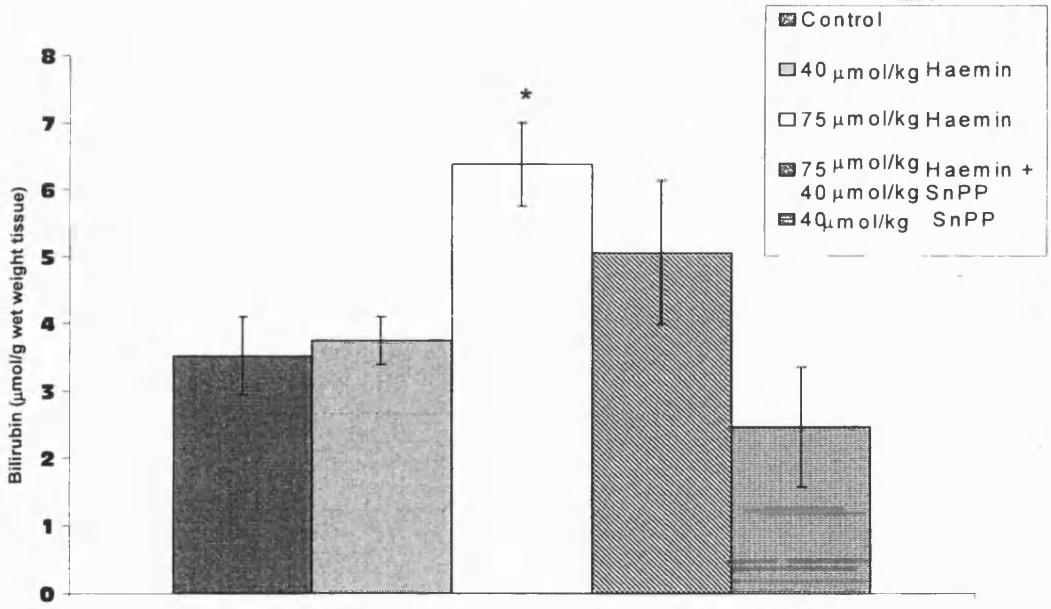


Figure 3.2.5

The effect of haemin and SnPP pre-treatment alone and in combination on basal tension (A) and tissue bilirubin levels (B) during reperfusion.

Rats were pre-treated with i.p. injections of saline, 40 µmol/kg and 75 µmol/kg haemin, plus 40 µmol/kg SnPP (in the presence of haemin or alone). Values are expressed as mean ± SEM, * where $p<0.05$ compared with control and # where $p<0.05$ compared with 75 µmol/kg haemin (n=5).

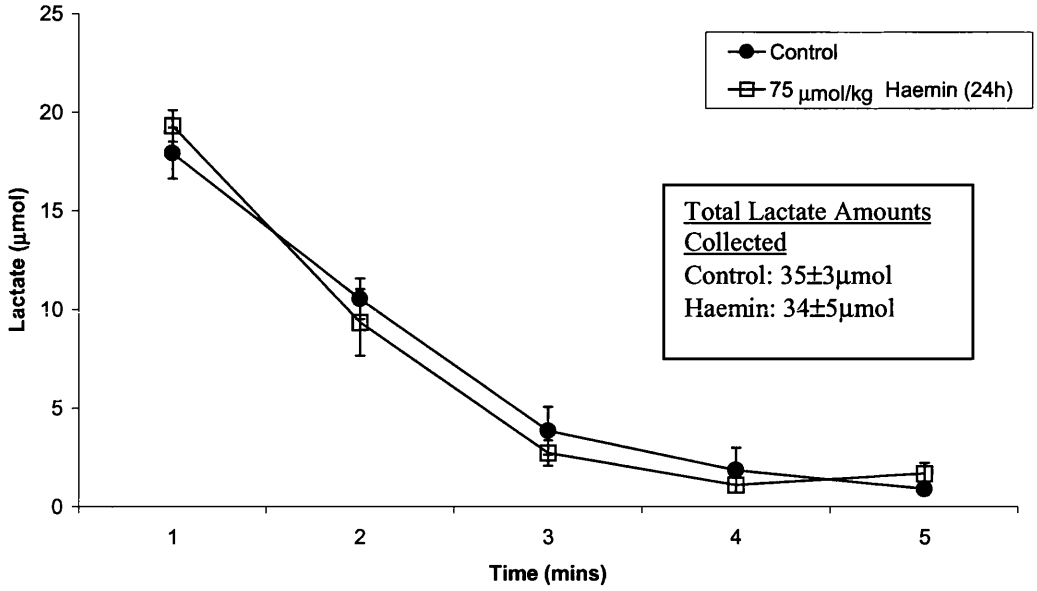


Figure 3.2.6
The effect of 75 µmol/kg haemin pretreatment (24h) on lactate washout in the I/R rat heart during the first 5 min of reperfusion.
Rats were pre-treated with saline or 75 µmol/kg. Values are expressed as mean ± SEM (n=5).

During ischaemia, lactate accumulates extracellularly as a result of increased anaerobic glycolysis and decreased wash out. Therefore, upon reperfusion lactate can be washed out, and measurement of this release is an indication of anaerobic glucose utilization. Hence, if pre-treatment with haemin were to protect the heart by increasing anaerobic glycolysis and ATP production, one might expect an increase in lactate washout during the initial stages of reperfusion. As shown by measuring the total amount of lactate, and also by its rate of removal, the actual data from control and haemin-treated hearts suggest that haemin does not affect lactate production, as demonstrated by the rate of removal and total lactate amounts. Therefore, the exact mechanism by which haemin can protect against I/R injury is as yet unclear, but it appears that it affects neither anaerobic glycolysis nor the production of lactate during ischaemia.

3.3 The effect of NOS inhibition on haemin-induced recovery in the I/R rat heart.

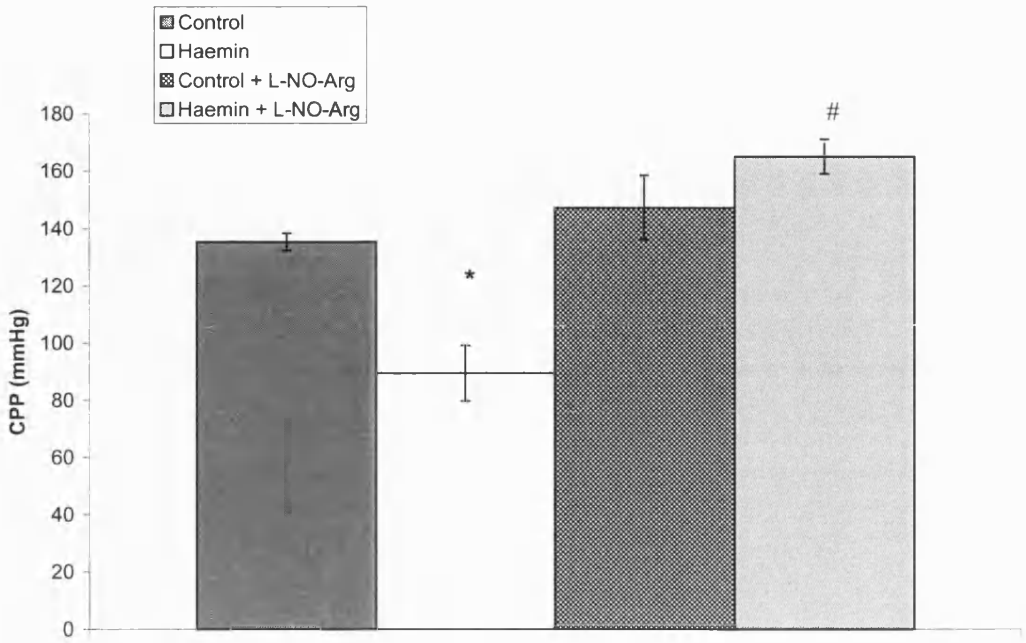
3.3.1 The effect of NOS inhibition in the pre-ischaemic heart.

We investigated the potential role of other vasoactive mediators in the response to haemin pre-treatment in the pre-and post-ischaemic heart. The results from the experiments in 3.2 indicate that SnPP does not completely inhibit the response to haemin on CPP in the pre- and post-ischaemic heart. This may be due to insufficient inhibition of HO-1 with the selected dose of haemin or the possible involvement of other factors in the CPP response to haemin. It is important to note that 3 of the total 8 hearts pre-treated with haemin and further perfused with L-NO-Arg exhibited ventricular fibrillation after the initial 5-10 min of exposure to L-NO-Arg.

L-NO-Arg was introduced to the perfusate after the initial 10 min of perfusion. Interestingly, L-NO-Arg did not have any significant effect on CPP in control hearts (Figure 3.3.1A). This may suggest that there is very little basal NO production in this system. The addition of 100 μM L-NO-Arg significantly increased CPP in haemin–treated tissues compared with those treated with haemin alone ($p<0.05$, $n=5$). The addition of L-NO-Arg prior to ischaemia did not have any significant effect on LVDT compared with control or haemin treatment after 30 min stabilization (figure 3.3.1B). As L-NO-Arg did not alter CPP it is unlikely that changes in LVDT are caused by vasodilation.

Inhibition of NO production by L-NO-Arg does not have any significant effect on heart rate compared with control (Figure 3.3.2A). Therefore, these data parallel the results in figure 3.3.1B, where L-NO-Arg had no effect on contractility. This is in agreement with the data in figure 3.3.2A, as NO does not appear to be involved in the control of contractility and heart rate in control or haemin treated hearts perfused at constant-flow. The inhibition of NOS in control and haemin-treated hearts did not significantly affect basal tension compared with control and haemin-treated hearts respectively (Figure 3.3.2B). This suggests that L-NO-Arg does not affect the stability of the heart prior to the induction of ischaemia.

(A) CPP



(B) LVDT

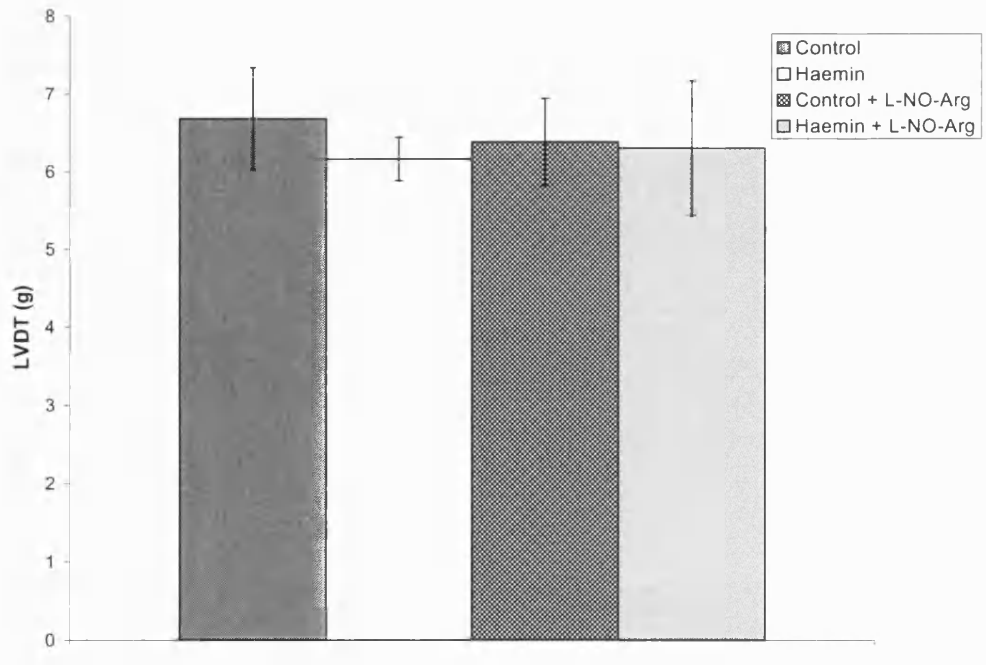
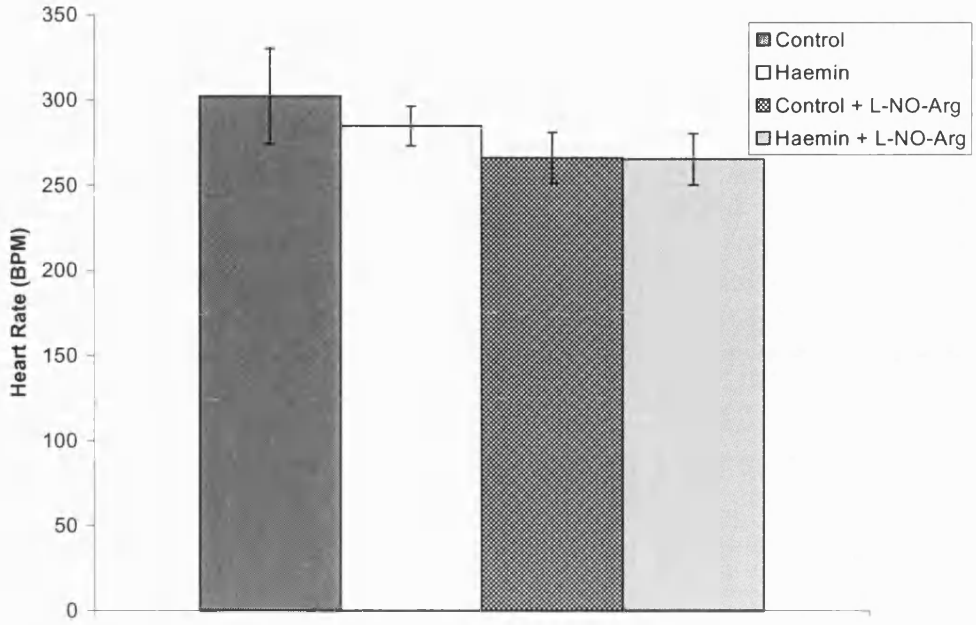


Figure 3.3.1

The effect of 100μM L-NO-Arg on pre-ischaemic CPP (A) and LVDT (B) in control and haemin pre-treated rat hearts perfused at constant-flow.

Rats were treated with 75μmol/kg haemin and saline (24hours) and L-NO-Arg was introduced to perfusate after initial 10 min of perfusion. Values are measured at the end of the 30min stabilization period and expressed as the mean ± SEM. * $p<0.05$ compared with control and # $p<0.05$ compared with haemin alone (n=5).

(A) Heart Rate



(B) Basal Tension

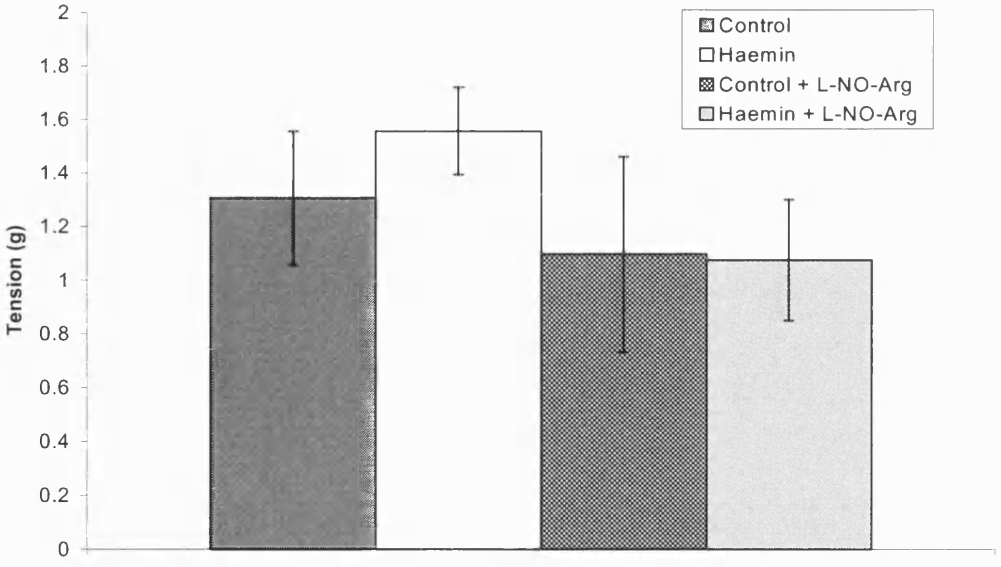


Figure 3.3.2

The effect of 100μM L-NO-Arg on pre-ischaemic heart rate (A) and basal tension (B) in control and haemin-treated rat hearts.

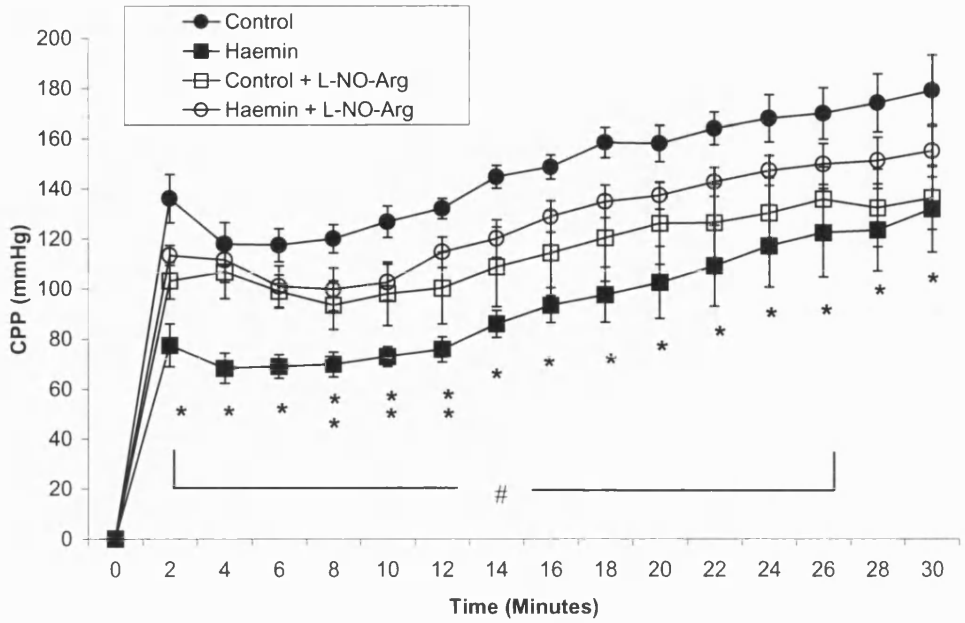
Rats were pre-treated with saline or 75 μmol/kg haemin (24hours) and L-NO-Arg was introduced to perfusate after the initial 10 min perfusion. Values were measured at the end of the 30min stabilization period and are expressed as mean ± SEM (n=5).

3.3.2 The effect of NOS inhibition on the recovery of cardiac function after I/R in haemin-treated rat hearts.

The addition of L-NO-Arg significantly reduced CPP in control hearts (see Figure 3.3.3A, where t= 6-10 min of reperfusion). Haemin–treated hearts exhibit a significant reduction in CPP post-ischaemia. In the presence of L-NO-Arg, the reduction in CPP produced by haemin treatment is significantly attenuated ($p<0.05$, where t= 2-26 min, n=5) to levels similar to those of control samples. Consequently, there appears to be an element of NO involvement in the vasodilator response to HO-1 in the rat heart perfused at constant-flow. The effect of L-NO-Arg on the haemin-induced CPP was reduced after 26 min of reperfusion, suggesting that the mediator(s) involved in the response to haemin pre-treatment were washed out or had ceased to be produced by this stage of the reperfusion period.

Inhibition of NOS did not have a significant effect on recovery of contractility after I/R compared with control. Furthermore, inhibition of NOS did not significantly affect the improved recovery of LVDT associated with haemin pre-treatment, although there are large error bars (Figure 3.3.3B). In conclusion, NOS inhibition with 100 μM L-NO-Arg does not prevent the protection of LVDT during reperfusion induced by haemin pre-treatment. There is a significant increase in CPP in haemin-treated hearts in the presence of L-NO-Arg, which suggests that although NO is involved in the vasodilator response to haemin, this effect may not influence the degree of recovery of LVDT.

(A) CPP – during reperfusion



(B) Recovery of LVDT

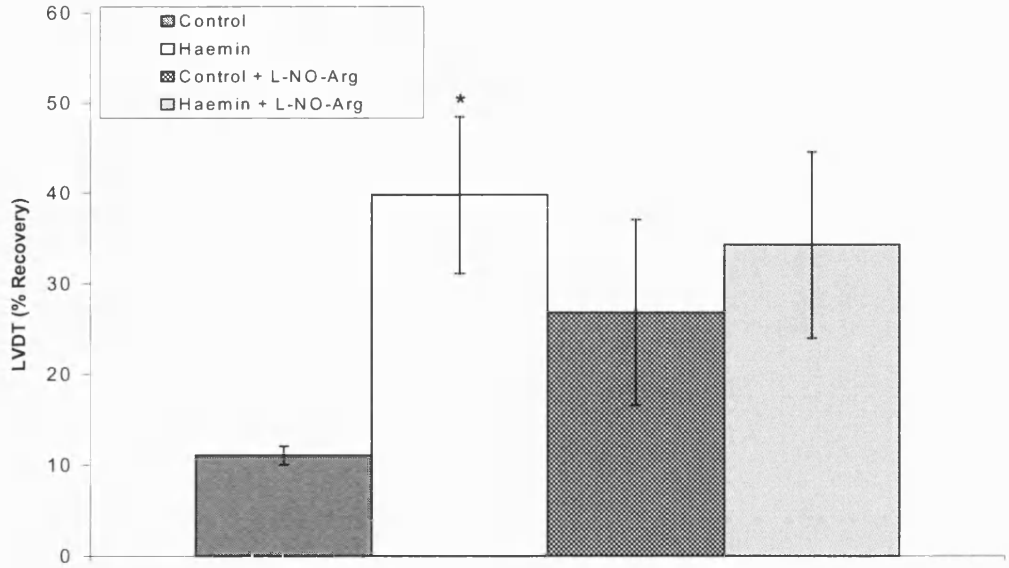
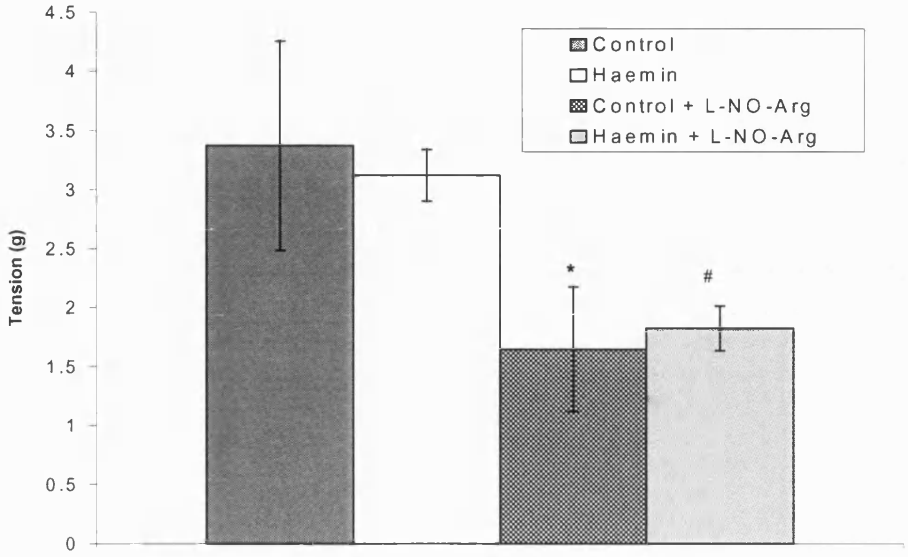


Figure 3.3.3

The effect of 100μM L-NO-Arg on CPP (A) and recovery of LVDT (B) post-I/R in control and haemin pre-treated rat hearts.

Rats were pre-treated with saline or haemin (24hours). Values are expressed as mean ± SEM. * where $p<0.05$ compared with control, # $p<0.05$ Haemin + L-NO-Arg compared with haemin alone and ** L-NO-Arg compared with control (n=5).

(A) Basal tension – during reperfusion.



(B) Tissue bilirubin production

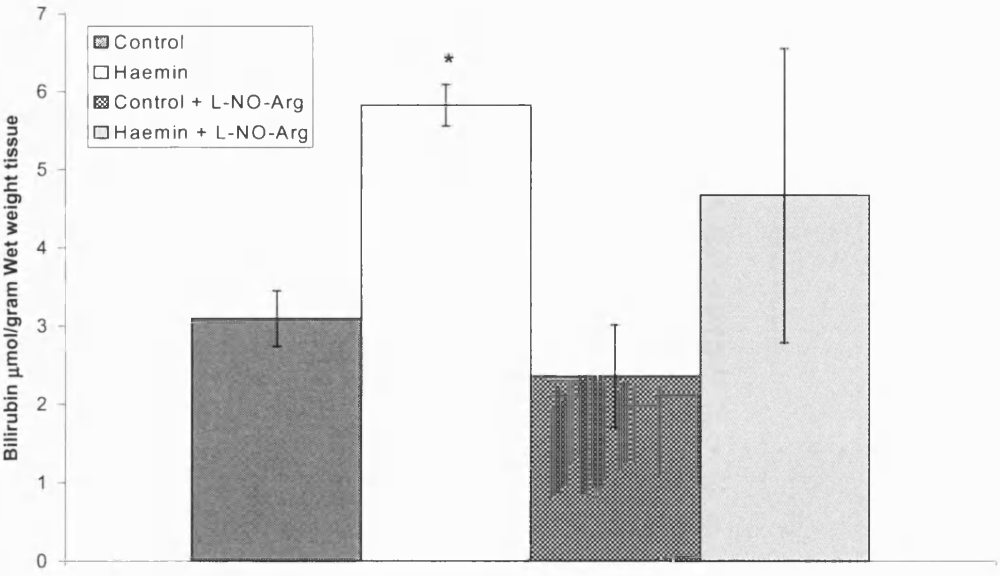


Figure 3.3.4

The effect of 100µM L-NO-Arg on post-ischaemic basal tension (A) and tissue bilirubin production (B) in control and haemin-treated rat hearts.
Rats were treated with saline or 75µmol/kg haemin (24hours). Values are expressed as mean ± SEM, where * $p<0.05$ compared with control and # $p<0.05$ compared with haemin (n=5).

After I/R, L-NO-Arg did not significantly affect basal tension in control or haemin-treated hearts (Figure 3.3.4A). This suggests that inhibition of NOS does not have a significant effect on the induction of Ca^{2+} overload after I/R. Furthermore, these data, in combination with figure 3.3.3A, suggest that L-NO-Arg does not inhibit the protection conferred by haemin pre-treatment on I/R injury, as it does not significantly increase the basal tension. Calculation of tissue bilirubin levels indicates that L-NO-Arg does not appear to affect the increase in tissue bilirubin levels induced by haemin pre-treatment, although large errors are apparent (Figure 3.3.4B). This is in association with the finding that NOS inhibition does not significantly affect the degree of recovery from I/R (Figure 3.3.3B). The results from figure 3.3.5 indicate that inhibition of NOS has no significant effect on lactate washout after I/R as measured after each minute, compared with control or haemin hearts, but there is a significant reduction in total lactate release in the presence of L-NO-Arg ($p<0.05$). Hence, inhibition of NO production significantly reduces the anaerobic glucose metabolism of the tissue after I/R, as shown by the reduction of total lactate levels.

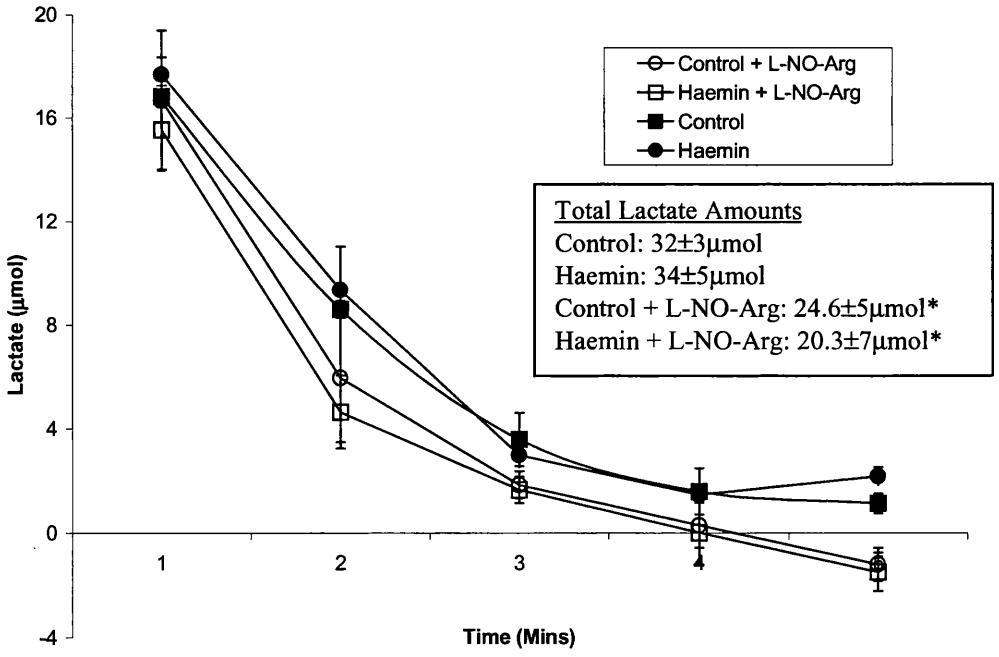


Figure 3.3.5

The effect of 100µM L-NO-Arg on lactate washout in the I/R rat heart.
Rats were pre-treated with saline or 75 µmol/kg haemin (24hours). The tissues were exposed to a 20 min ischaemic period and upon reperfusion the perfusate was collected for the initial 5 min for lactate analysis. Mean ± SEM, * $p<0.05$ compared with control. Note: a negative value was observed at t=5 min in the presence of L-NO-Arg.

3.4 Determination of the influence of COX products on haemin-induced recovery of cardiac function post I/R.

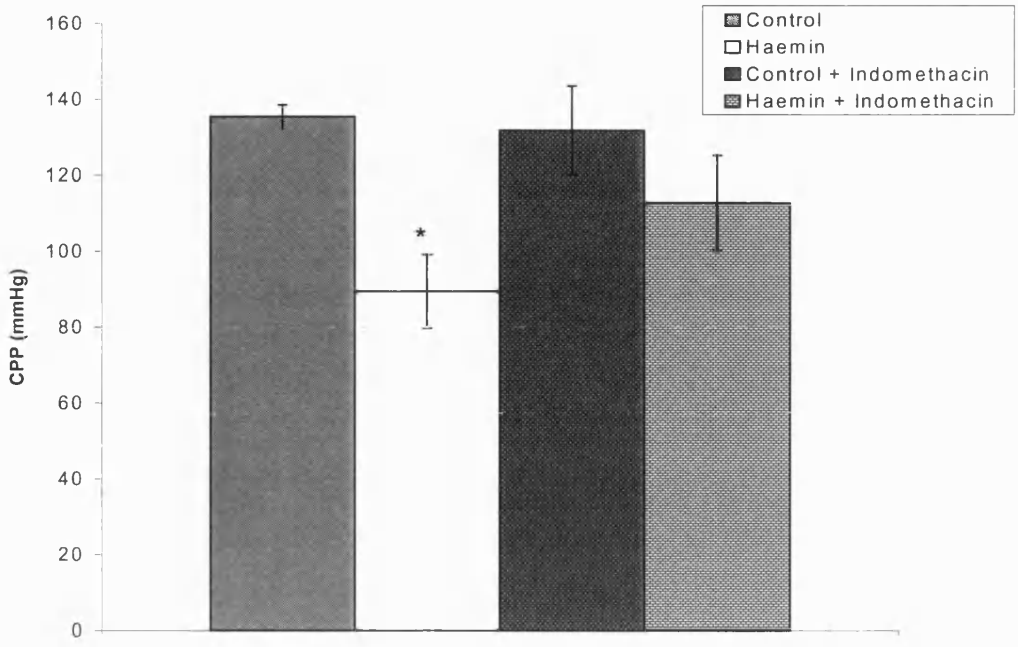
3.4.1 The effect of COX inhibition in the pre-ischaemic rat heart.

The initial experiments reported in section 3.3 suggested that factors other than NO might be involved in the vasodilator response observed in pre- and post-ischaemic rat hearts treated with haemin. The following experiment investigated the possible effect of COX products on the response to haemin pre-treatment in the pre- and post-ischaemic rat heart.

Indomethacin treatment did not significantly affect CPP in pre-ischaemic control hearts. However, there did appear to be a small reversal of the haemin-induced response in the presence of indomethacin. Furthermore, the use of indomethacin as a COX inhibitor did not induce any significant effect on the dilator response to haemin in the pre-ischaemic heart (Figure 3.4.1A). In contrast to the CPP data, haemin-treated hearts perfused with 10 μM indomethacin exhibited a significant increase in LVDT compared with hearts treated haemin alone, ($p<0.05$ and $n=5$, Fig 3.4.1B). This suggests that COX products may be involved in the control of contractility in haemin-treated hearts. As there was no significant effect on CPP, this is unlikely to influence the force of contraction.

Indomethacin did not significantly affect HR in control hearts. COX inhibition after haemin pre-treatment significantly reduced heart rate compared with control and haemin-treated hearts ($p<0.05$, $n=5$, Figure 3.4.2A). The reduction in heart rate may be a response to an increase in contractility (Figure 3.4.1B) or vice versa. The data in figure 3.4.2B indicate that inhibition of COX using indomethacin did not significantly affect basal tension in the pre-ischaemic heart compared with control and haemin-treated hearts.

(A) CPP



(B) LVDT

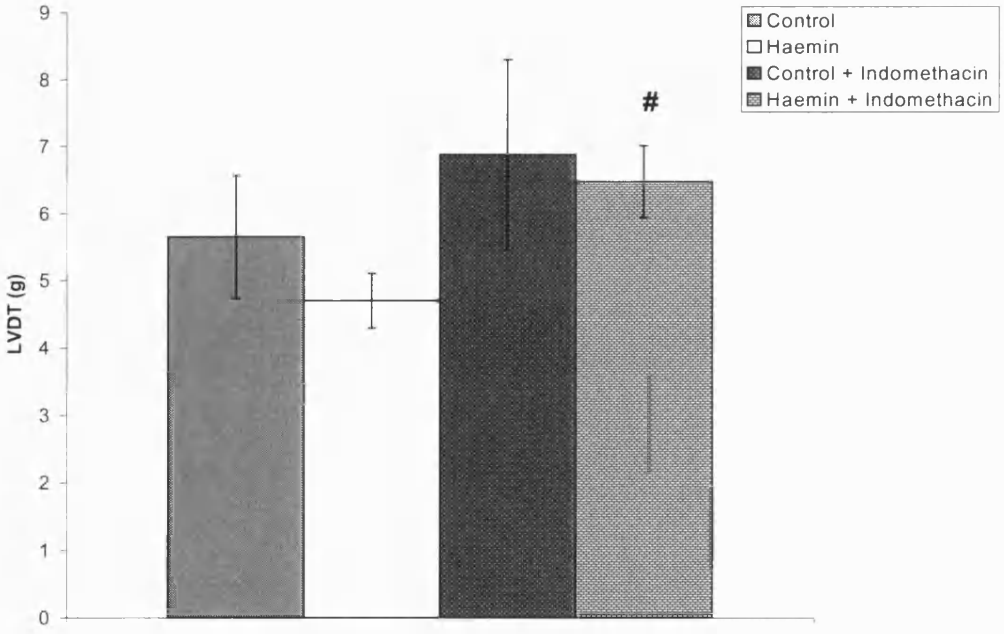
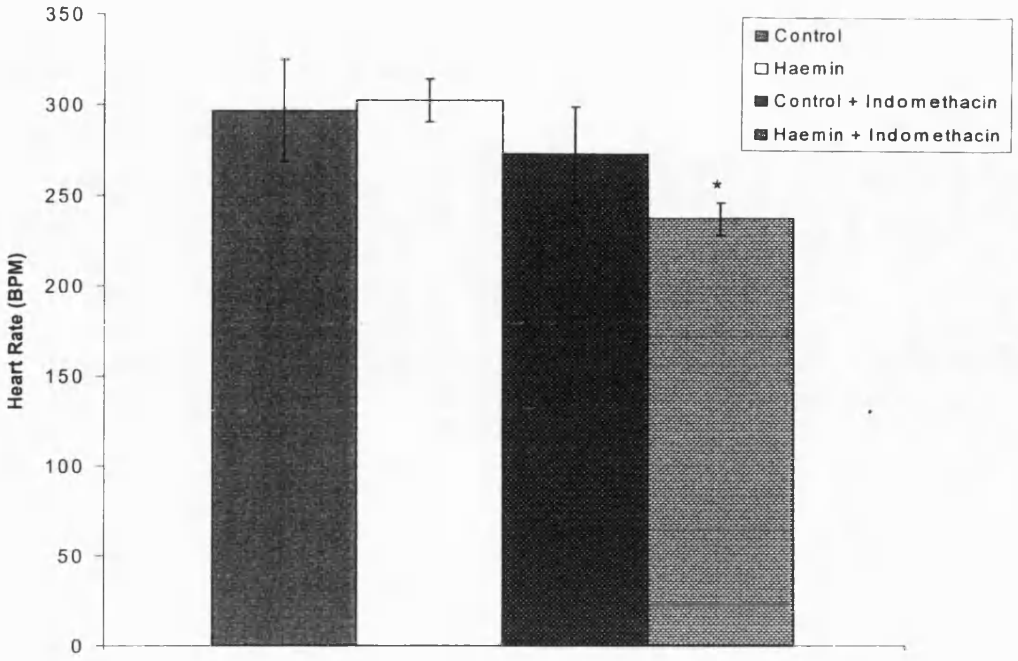


Figure 3.4.1

The effect of 10μM indomethacin on pre-ischaemic CPP (A) and LVDT (B) in control and haemin-treated rat hearts.

Rats were pre-treated with saline or 75 μmol/kg haemin (24hour). Values were measured at the end of the 30 min stabilization period and are expressed as mean ± SEM, * $p<0.05$ compared with control and # $p<0.05$ compared with haemin alone (n=5).

(A) Heart rate



(B) Basal tension

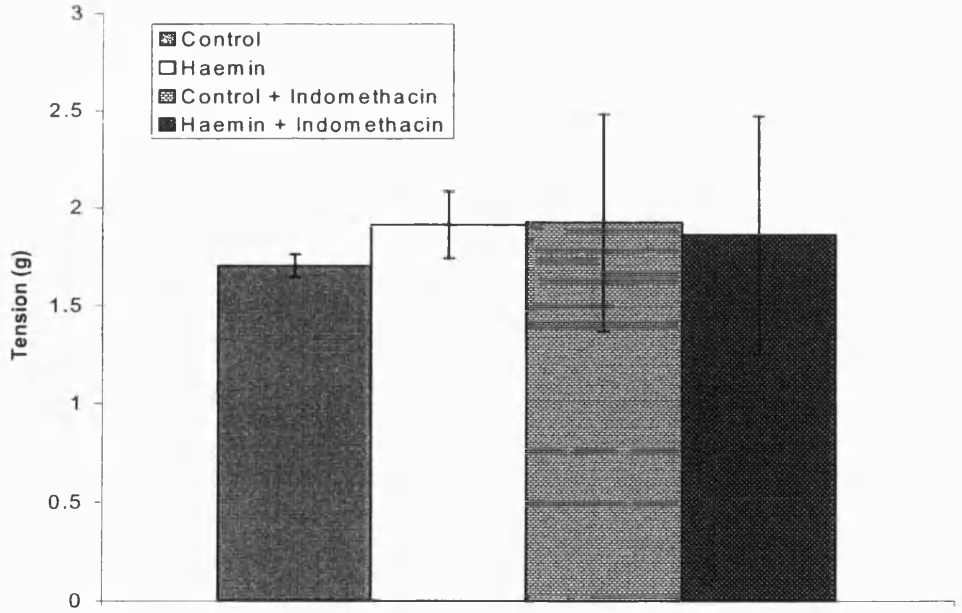


Figure 3.4.2

The effect of 10μM indomethacin on pre-ischaemic heart rate (A) and basal tension (B) in control and haemin-treated rat hearts.

Male wistar rats were pre-treated with saline or 75 μmol/kg haemin (24hour). Values were measured at the end of 30 min stabilization and are expressed as mean ± SEM, where * $p<0.05$ compared with control hearts and haemin treated hearts.

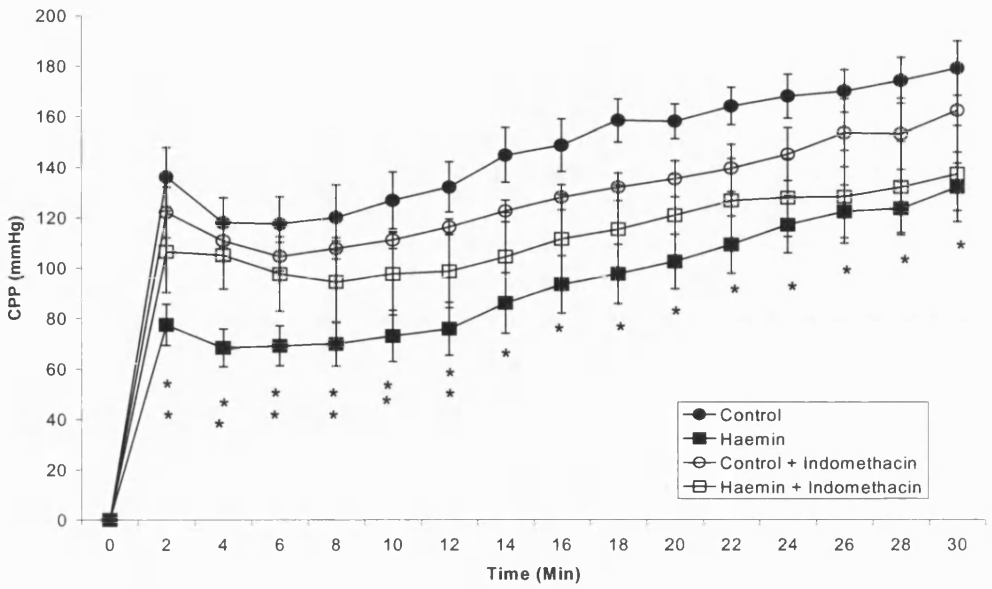
3.4.2 The effect of COX inhibition on recovery of cardiac function in haemin-treated rat hearts.

After a 20 min period of global ischaemia, indomethacin significantly reduced the vasodilator component of the response to haemin pre-treatment during the first 12 min of reperfusion (Figure 3.4.3A). This is in contrast with the data recorded in the pre-ischaemic heart. Therefore, it is possible that COX products are only significantly involved in the vascular response to I/R, rather than under basal conditions, in this system. However, the profile is similar to that observed in the presence of L-NO-Arg (Figure 3.3.3A), where the significant effect is the inhibition of a vasodilator mediator involved in the response to haemin pre-treatment during the early response to I/R.

Indomethacin had no significant effect on the recovery of control hearts, suggesting that this is a haemin-induced response, and that COX products may not be involved in the endogenous response to ischaemic insult in this system. The inhibition of COX significantly abrogated the improved recovery of contractility after I/R conferred by haemin (Figure 3.4.3B). These data suggest that COX products are involved in the recovery of contractility conferred by haemin pre-treatment. This confirms the pre-ischaemic data, as COX products appear to be involved in the regulation of contractility in haemin-treated hearts. Indomethacin treatment did not significantly affect the basal tension compared with either haemin or control (Figure 3.4.4A).

Indomethacin treatment did not significantly affect tissue bilirubin levels in control hearts (Figure 3.4.4B). The addition of indomethacin to the perfusate of haemin treated hearts significantly increased tissue bilirubin levels compared to control and haemin alone. Hence, these data may implicate the involvement of COX products in the regulation of tissue bilirubin levels in haemin-treated hearts. These data do not directly correlate with those seen in figure 3.4.3B, where indomethacin significantly reduces the recovery of cardiac contractility conferred by haemin pre-treatment.

(A) CPP – during reperfusion



(B) Recovery of LVDT

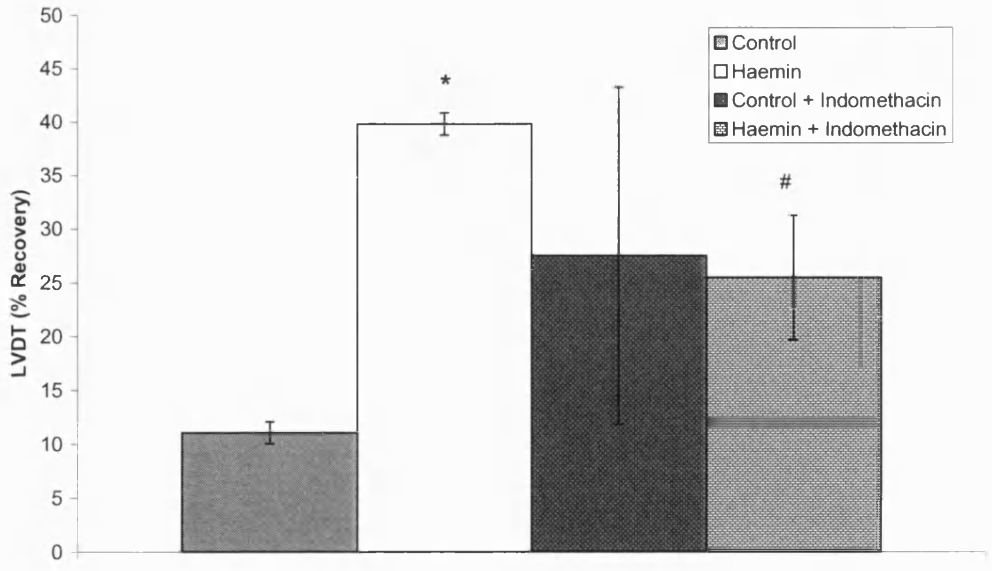
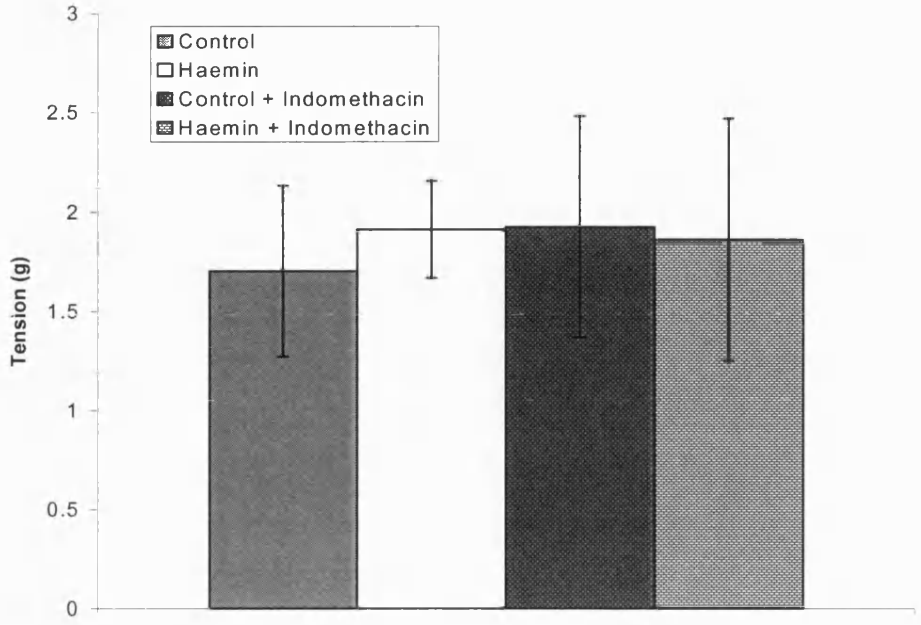


Figure 3.4.3

The effect of COX inhibition using 10μM indomethacin on post-ischaemic CPP in control and haemin–treated rat hearts.

Rats were pre-treated with saline or 75μmol/kg haemin (24hour). Values are expressed as mean ± SEM. * where $p<0.05$ compared with control or # where $p<0.05$ compared with haemin alone (n=5). Note: some errors may within the symbol.

(A) Basal tension during reperfusion



(B) Tissue bilirubin production

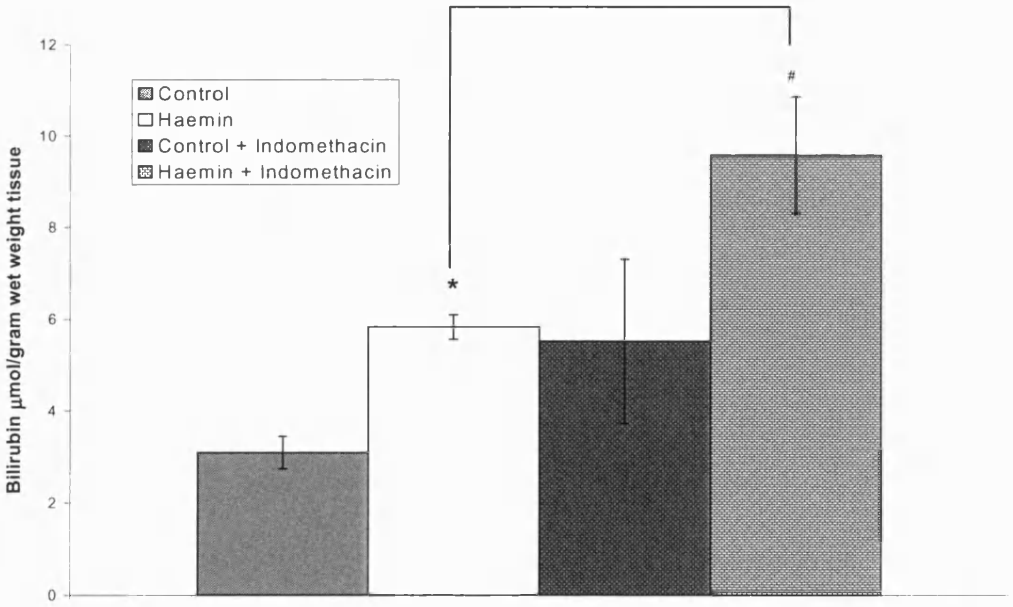


Figure 3.4.4

The effect of COX inhibition using 10µM indomethacin on basal tension (A) and tissue bilirubin levels (B) after I/R in control and haemin –treated rat hearts.

Rats were treated with saline or 75 µmol/kg haemin (24hour). Values are measured at the end of 30 min reperfusion and expressed as mean ± SEM. * $p<0.05$ compared with control and # $p<0.05$ compared with haemin (n=5).

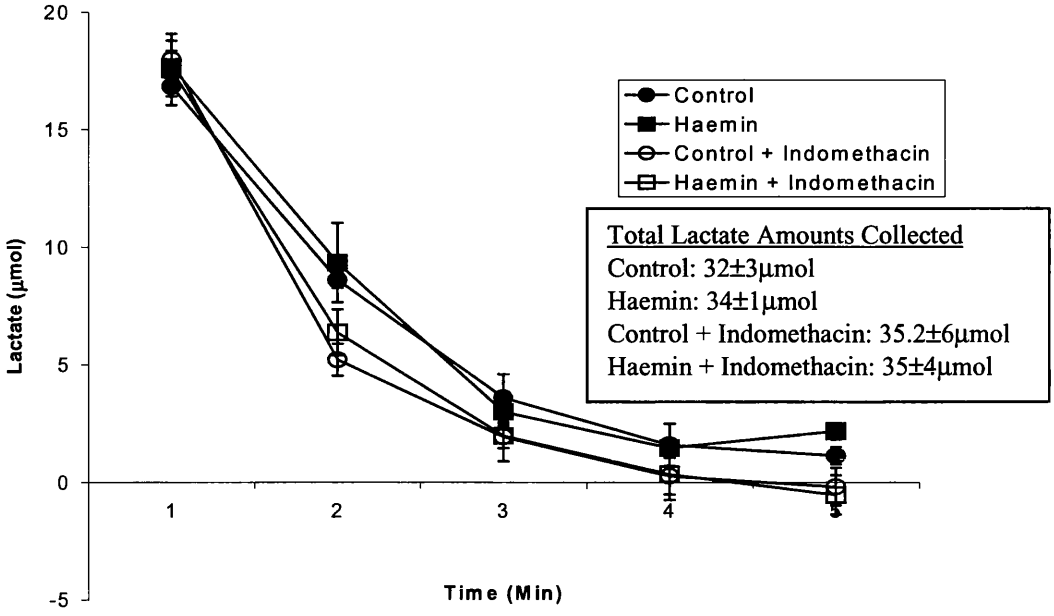


Figure 3.4.5
The effect of COX inhibition using 10µM indomethacin on lactate wash-out during the first 5 min of reperfusion in control and haemin-treated rat hearts.
Male wistar rats were treated with saline or 75 µmol/kg haemin (24hour). The perfusate was collected during the initial 5 min for the calculation of lactate content. Values are expressed as mean ± SEM (n=5). Note: some errors fall within symbol size.

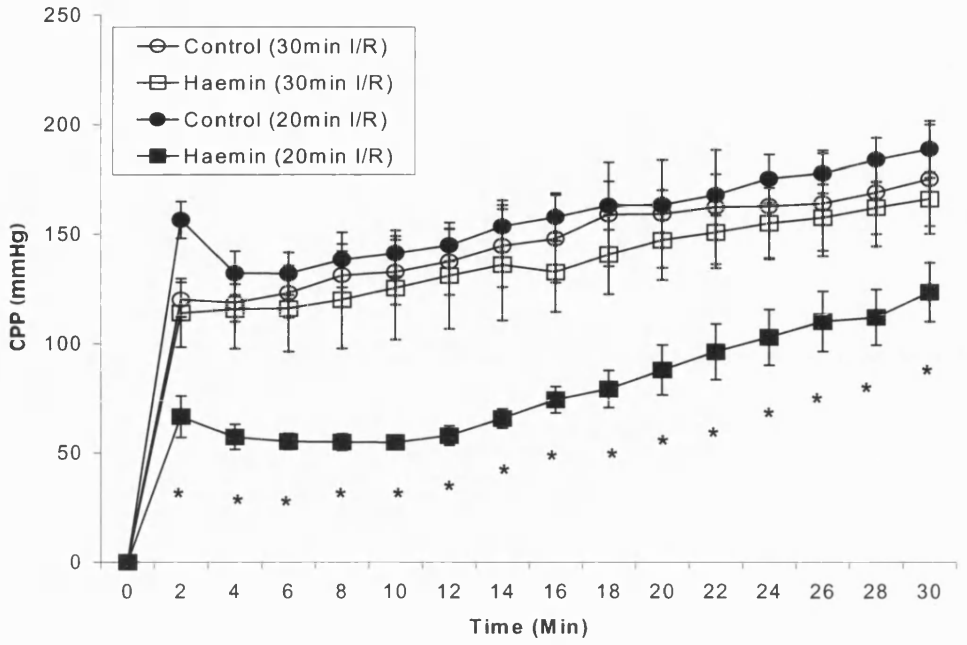
The addition of indomethacin did not significantly affect the rate of lactate wash-out or total lactate release during reperfusion after an ischaemic insult of 20 min (Figure 3.4.5). The recovery of cardiac function throughout the series of data does not appear to be accompanied by any change in lactate levels.

3.5. The effect of increasing ischaemic period on haemin-induced recovery of cardiac function after 30mins I/R.

The effect of haemin pre-treatment on the recovery of cardiac function after an ischaemic insult of 30 min was investigated to determine whether the protective effect post I/R is dependent upon the length of ischaemic insult and thus to answer the following questions: firstly, does the dose of haemin used offer sufficient protection after a more severe ischaemic injury where it is possible that irreversible injury may be caused? And secondly, could we repeat the result described by Clark et al. (2000), from which our dose of haemin was selected, where haemin offered significant protection from 30 min I/R.

Interestingly, the CPP measured in control hearts is not different after 20 and 30min ischaemia (Figure 3.5.1A), whereas the increase in ischaemic insult significantly increases CPP in haemin-treated hearts compared with 20 min ischaemia. Therefore, it is possible that increasing ischaemia may affect the vascular responsiveness of the tissue, as illustrated by the abolition of the vasodilator response to haemin treatment. In addition, the recovery of contractility does not significantly differ in control hearts exposed to 20 or 30 min ischaemia followed by reperfusion (Figure 3.5.1B), while the recovery of contractility induced by haemin pre-treatment is significantly reduced after increasing the ischaemic period to 30 min. The reperfusion period is the same in both experiments, suggesting that the destructive element must by produced during ischaemia. After 30 min I/R there was a significant increase in basal tension compared with 20 min I/R in control hearts (Figure 3.5.2A), although there was no significant difference in haemin-treated hearts after 20 or 30 min ischaemia. This indicates that increasing ischaemia in control hearts potentiates Ca^{2+} overload as measured by basal tension. This result is as expected, as basal tension and Ca^{2+} overload would be expected to increase as the duration of ischaemic period increases. The data in figure 3.5.2B shows that after 30 min ischaemia, haemin pre-treatment does not significantly increase tissue bilirubin levels compared with control. It can be postulated that the reduced recovery of cardiac function after a lengthened ischaemic period may be due to a reduction in the levels of bilirubin.

(A) CPP during reperfusion



(B) Recovery of LVDT

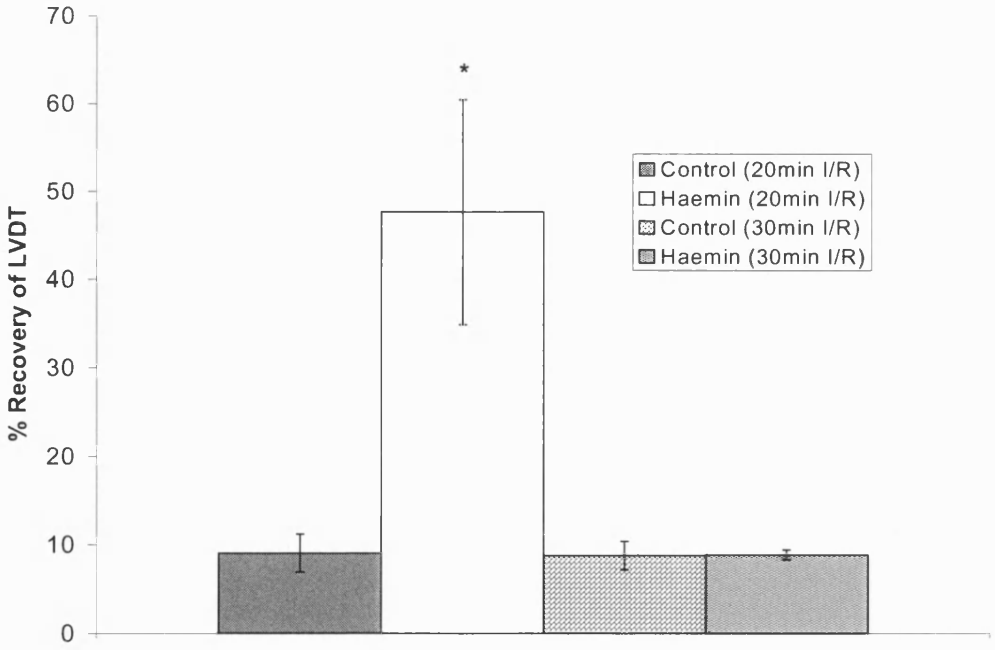
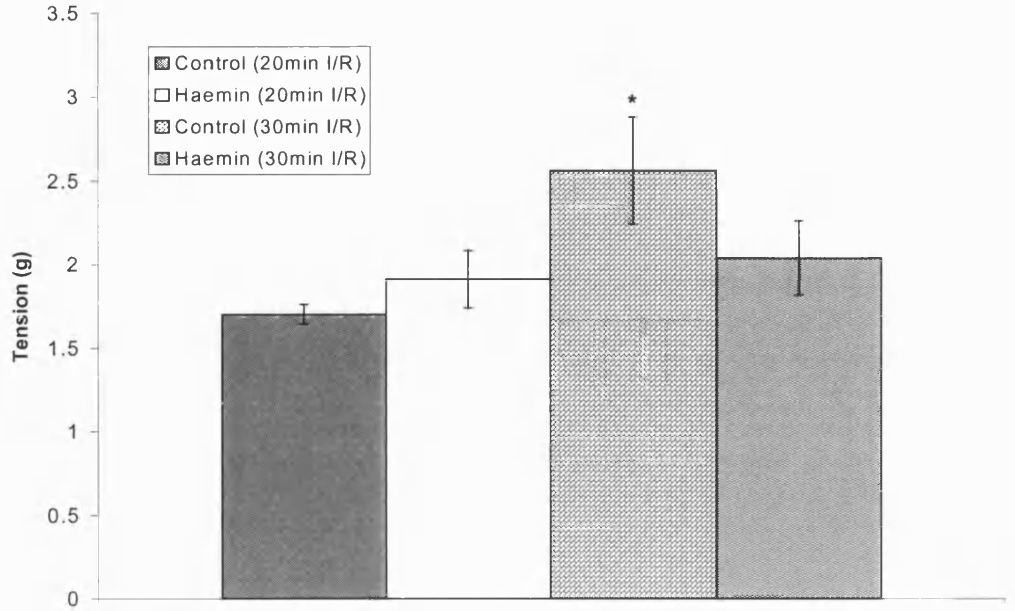


Figure 3.5.1

The effect of increasing the ischaemic period on CPP (A) and recovery of LVDT (B) during reperfusion after a 20 or 30 min ischaemic insult in control and haemin-treated hearts.

Male wistar rats were pre-treated with saline or 75 µmol/kg Haemin (24hour). Values are expressed as the mean ± SEM, * where $p<0.05$ compared with control (n=5). Note: some error bars fall within the size of the symbol.

(A) Basal tension



(B) Tissue bilirubin production

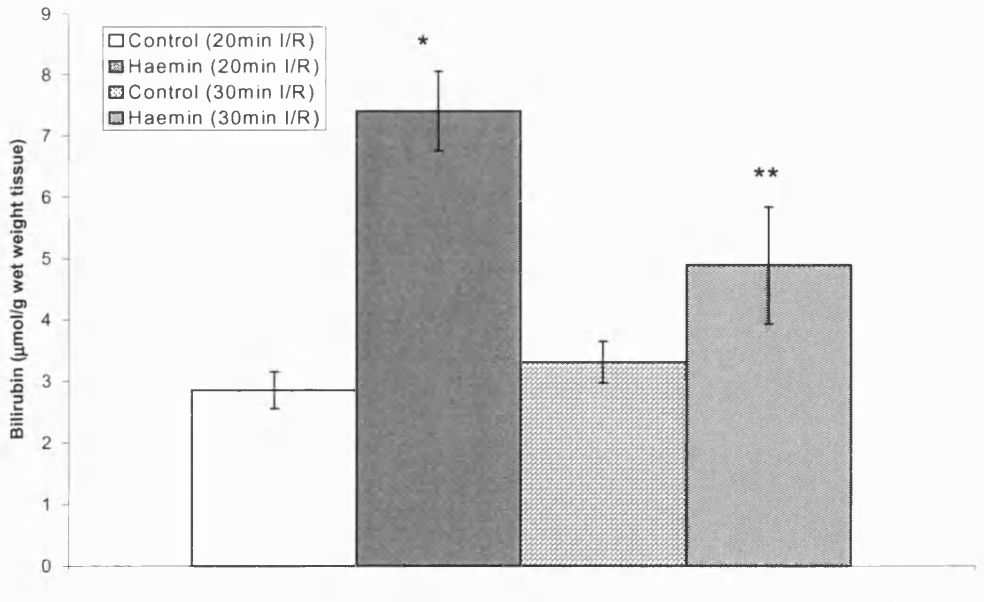


Figure 3.5.2

The effect of increasing ischaemic period on basal tension (A) and tissue bilirubin levels (B) in control and haemin-treated hearts.

Male wistar rats were treated with saline or 75 µmol/kg haemin (24hour) prior to removal of heart. Values are expressed as mean ± SEM. * where $p<0.05$ compared with control and ** $p<0.05$ compared with haemin (20min I/R) (n=5).

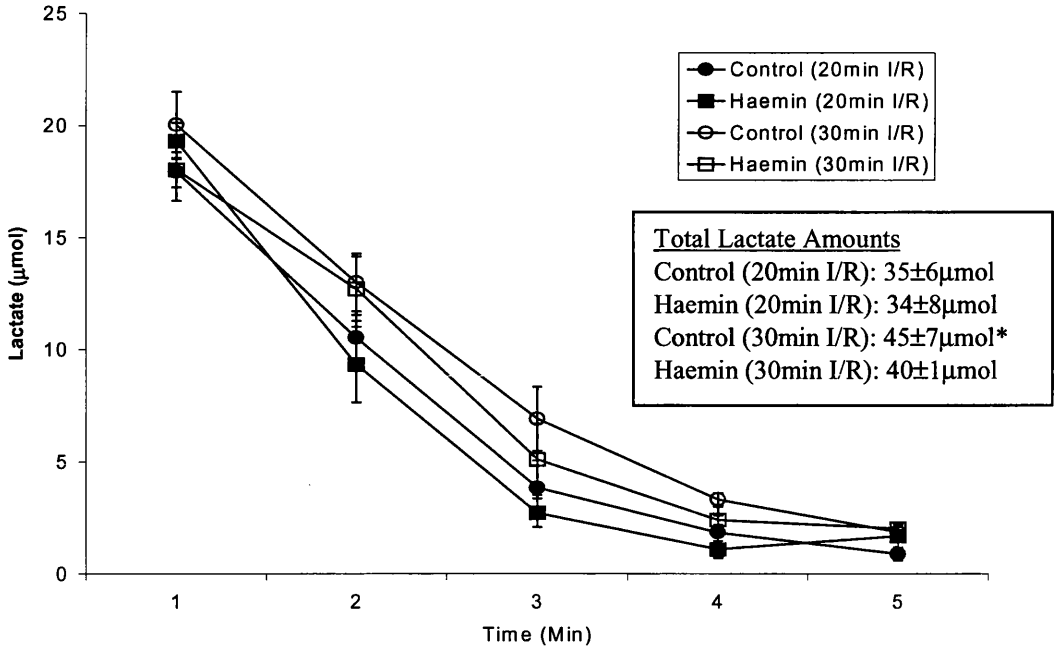


Figure 3.5.3

The effect of increasing ischaemic period on lactate washout during the first 5 min of reperfusion in control and haemin-treated hearts.

Male wistar rats were treated with saline or 75 µmol/kg haemin (24hour) prior to removal of heart. Values are expressed as mean ± SEM (n=5) and *$p<0.05$ compared with control hearts after 20mins ischaemia. Note: some errors fall within the symbol size.

The increase in duration of ischaemic period did not significantly affect the washout of lactate over each minute during the first 5 min of reperfusion in either treatment group, but there was a significant increase in the total lactate amounts present in control hearts after 30 min I/R. Therefore, it is possible that haemin treatment may not affect anaerobic glycolysis as there is no increase in lactate production after 30 min I/R.

3.6 Chapter 3: Summary

- 75μmol/kg haemin pre-treatment successfully increased HO-1 protein expression in the rat heart
- Haemin pre-treatment significantly reduced CPP in the pre-ischaemic rat heart; this is indicative of a vasodilator response.
- SnPP alone significantly increased contractility and heart rate and SnPP significantly reduced basal tension in the presence or absence of haemin.
- Haemin pre-treatment significantly increased recovery of cardiac function after 20 min ischaemia and 30min reperfusion and also produced a significant reduction in CPP during reperfusion.
- SnPP did not completely inhibit the effect haemin pre-treatment in either the pre- or post-ischaemic rat heart.
- Haemin induced recovery of contractility was linked to an increase in tissue bilirubin content.
- Increasing ischaemic period to 30 min significantly reduced recovery in control and haemin-treated hearts. This was associated a significant increase in tissue bilirubin content of haemin-treated hearts and the complete abolition of the vasodilator response during reperfusion of haemin-treated hearts.
- L-NO-Arg significantly increased CPP in haemin-treated hearts, but did not affect CPP in control hearts.
- L-NO-Arg significantly increased CPP in haemin-treated hearts during the initial 20 min of reperfusion, but this was not accompanied by a reversal of the improved recovery of contractility in haemin-treated hearts.
- Indomethacin did not affect CPP but significantly increased contractility and reduced heart rate in pre-ischaemic haemin-treated hearts.
- After 20 min ischaemia, indomethacin significantly reduced recovery of contractility, although this was accompanied by a significant increase in tissue bilirubin levels.

CHAPTER 4:

THE ROLE OF HO-1 IN THE CONSTANT-PRESSURE-PERFUSED RAT HEART

CHAPTER 4: The role of HO-1 in the constant pressure perfused rat heart

4.1 The effect of haemin and SnPP pre-treatment on the pre-ischaemic rat heart perfused at a constant pressure of 70mmHg.

The following experiments were carried out to determine the vascular effect of haemin pre-treatment in the pre-and post-ischaemic rat heart perfused under conditions of constant pressure. The constant pressure heart model allows investigation of the effect of haemin pre-treatment on the control of coronary flow in a perfusion system where the endogenous coronary autoregulation mechanisms remain active.

4.1.1 A representative trace of the experimental procedure and observations / measurements made during procedure.

The representative traces demonstrated in figure 4.1.1A and 4.1.1B, indicate the procedure involved in the experiments at constant-pressure in haemin-treated and control hearts respectively. The hearts were set up and perfused at a constant flow of 10 ml/min for 10 min. The method of perfusion was then converted to a constant pressure of 70 mmHg. Contractility/LVDT (A), heart rate (B) and coronary flow rate (CFR) were measured throughout the experiment.

After a total of 30 min stabilization, the heart was subjected to 20 min zero-flow global ischaemia. In this section of the experiment, there was a gradual reduction in LVDT during the first 3 min. Upon reperfusion, there was a rapid return to a relatively strong force of contraction and HR within 1 min of reperfusion. HR showed a transient increase above the initial rate followed by a plateau after about 3-4 min. CFR increased upon reperfusion as part of the reactive hyperaemic response to ischaemia, but this also reached a plateau after about 10 min.

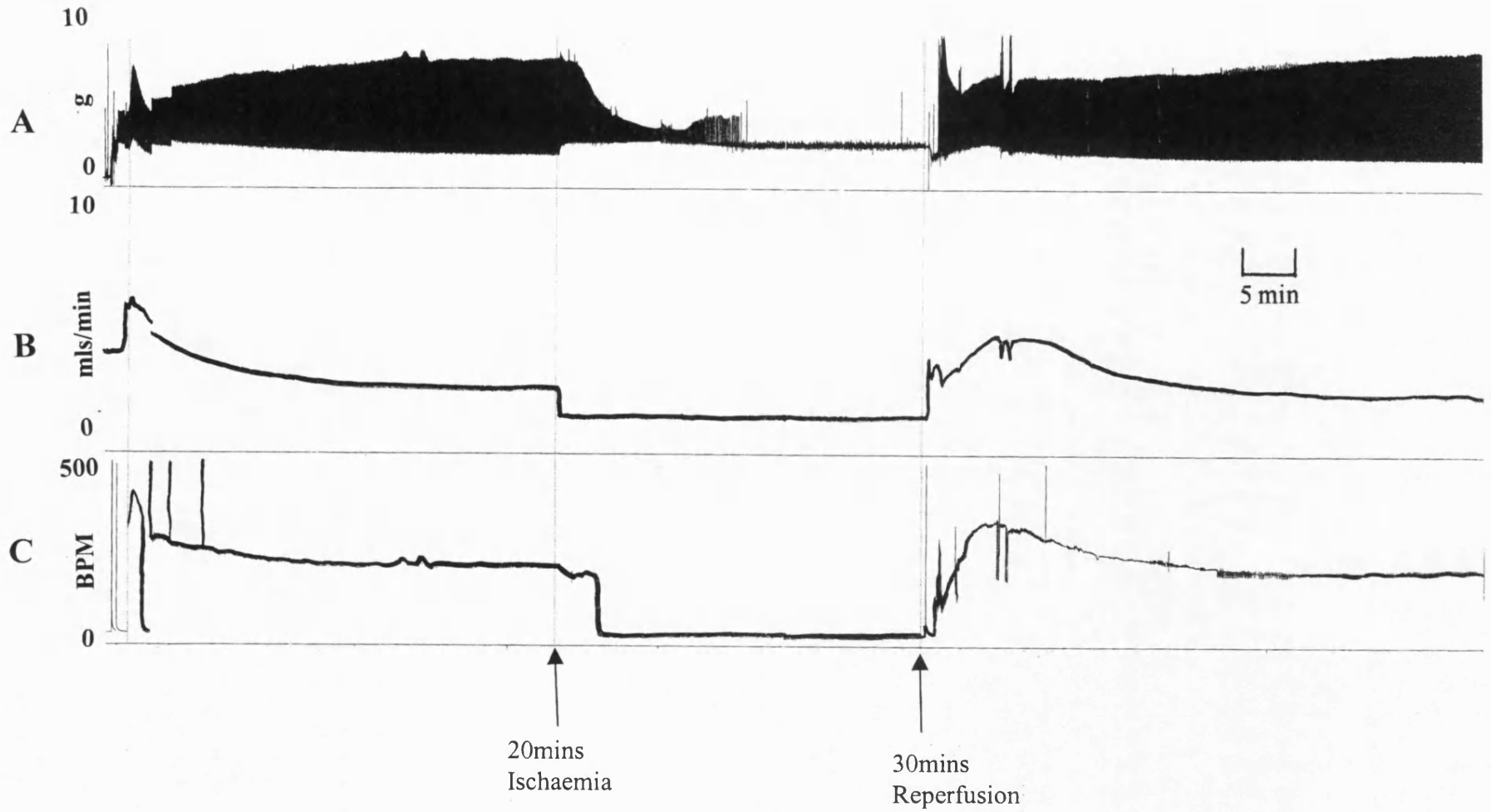


Figure 4.1.1A

This trace is representative of the experimental conditions and observations in a haemin-treated heart perfused at a constant-pressure of 70mmHg. The LVDT (section A), CFR (section B) and heart rate (section C) were measured throughout the initial stabilisation period, 20min ischaemic insult and 30 min reperfusion.

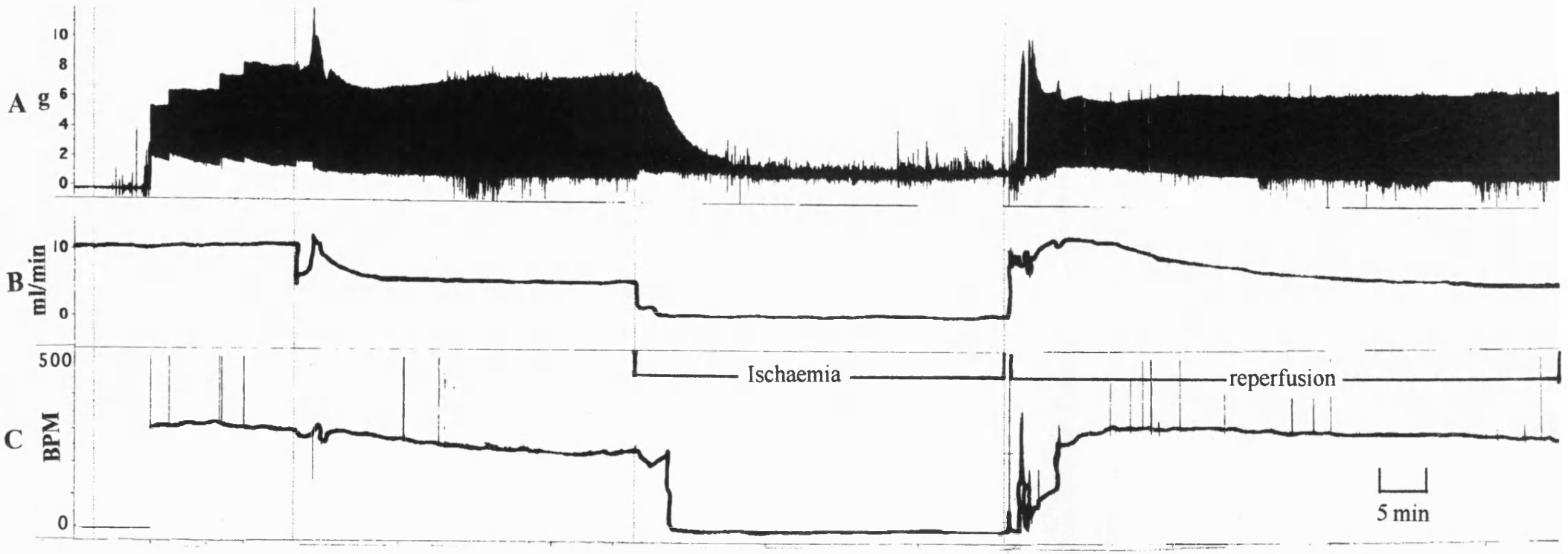


Figure 4.1.1B

This trace is representative of the experimental conditions and observations in a control heart perfused at a constant-pressure of 70mmHg. The LVDT (section A), CFR (section B) and heart rate (section C) were measured throughout the initial stabilisation period, 20min ischaemic insult and 30 min reperfusion.

HO-1 protein expression was induced in the rat heart using 75μmol/kg haemin (24 hour) as in the constant-flow perfusion experiments. The results were analysed using immunoblot analysis with an anti-rat HO-1 antibody.

As indicated in figure 4.1.2, 75μmol/kg haemin successfully increased HO-1 expression compared with control.

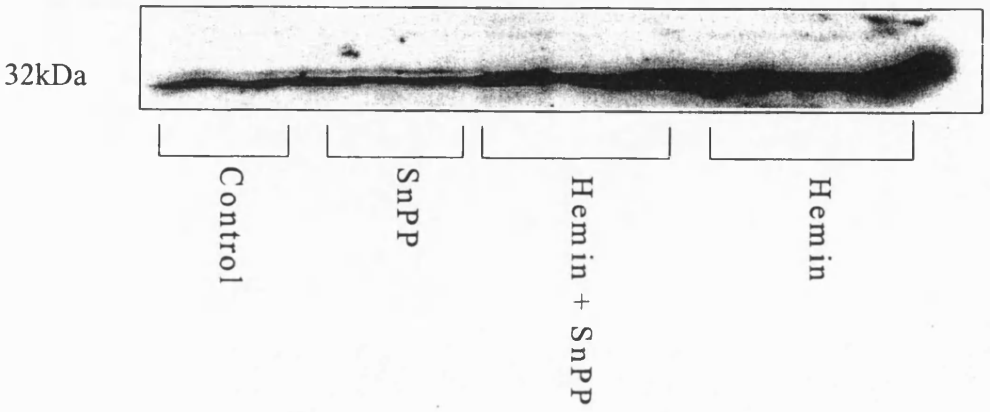
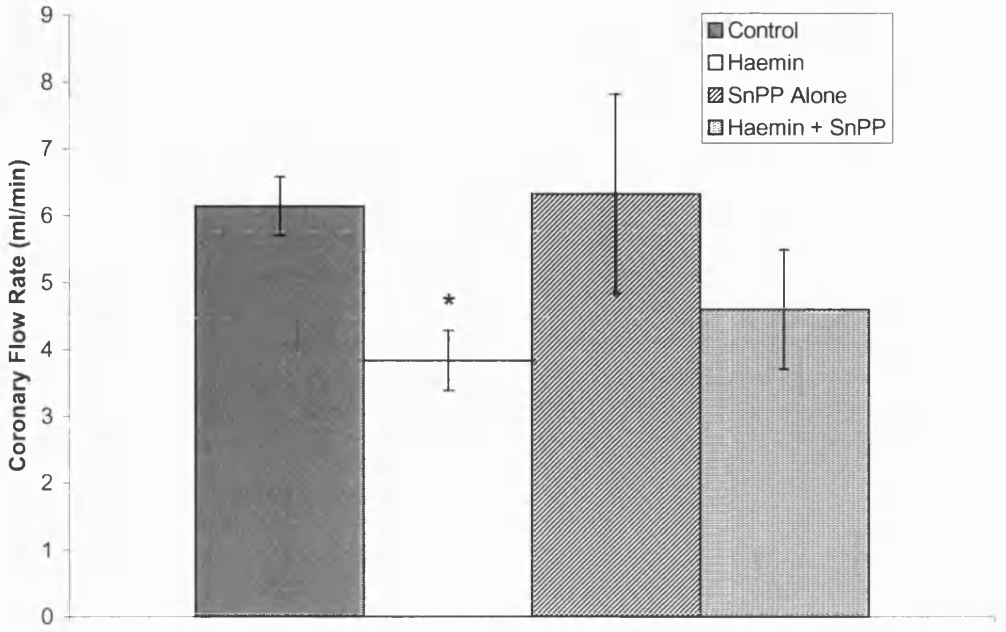


Figure 4.1.2
The effect of haemin pre-treatment on HO-1 expression in the rat heart perfused at a constant-pressure of 70mmHg.
Rats were treated with saline, 40μmol/kg SnPP (1 hour), 75μmol/kg haemin, and 75μmol/kg haemin + 40μmol/kg SnPP respectively. HO-1 protein expression was measured at the end of 20min ischaemia and 30 min reperfusion (n=2 in this particular blot).

4.1.2. The effect of haemin and SnPP pre-treatment on basal flow and LVDT in the pre-ischaemic rat heart perfused at 70 mmHg.

(A) CFR



(B) LVDT

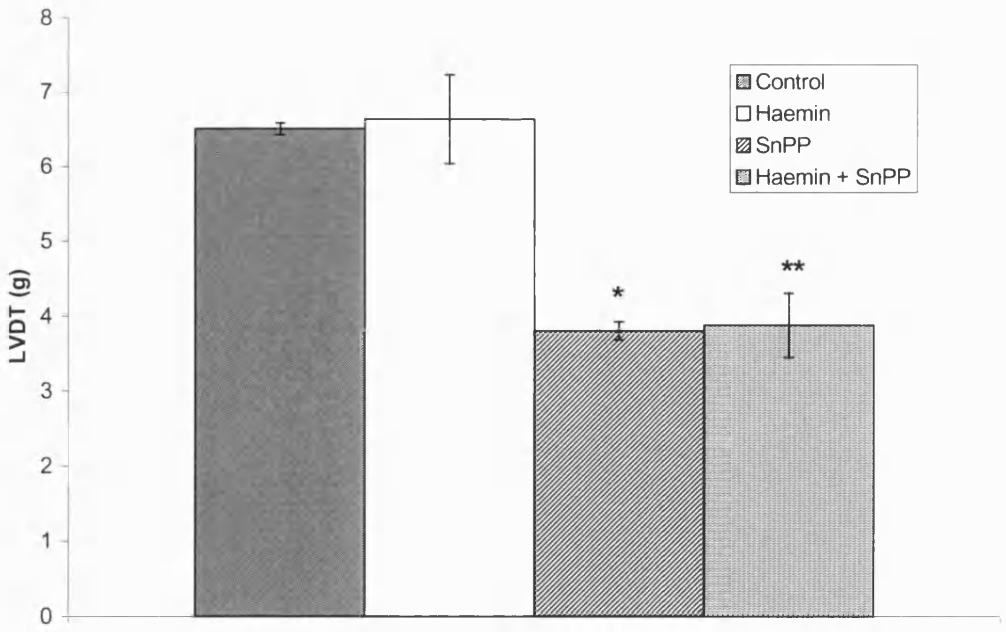
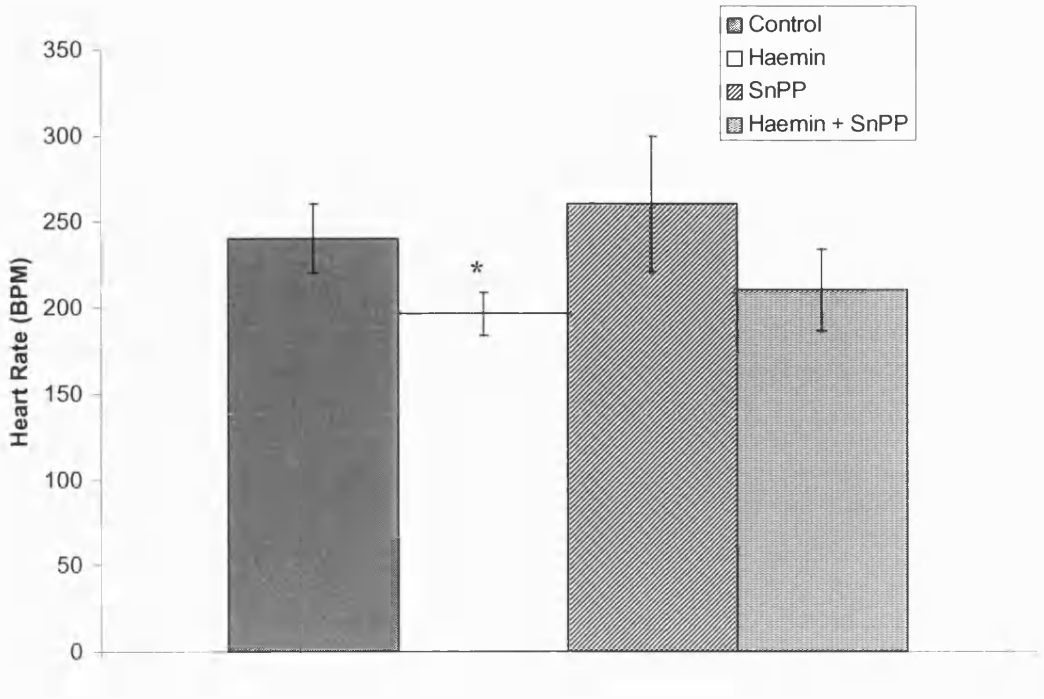


Figure 4.1.3
The effect of haemin and SnPP pre-treatment alone and in combination on CFR (A) and LVDT (B) in the pre-ischaemic rat heart perfused at a constant-pressure of 70mmHg.
Rats were pre-treated with saline, 75 µmol/kg haemin (24hour), 40 µmol/kg SnPP (1 hour) and haemin + SnPP. Values are expressed as mean ± SEM. * where $p<0.05$ compared with control and ** $p<0.05$ compared with haemin alone (n=5).

Haemin pretreatment significantly reduced CFR from 6.2 ml/min in control hearts to 3.8 ml/min in haemin-treated hearts ($p<0.05$, $n=5$) (Figure 4.1.3A). This effect is indicative of a vasoconstrictor response to haemin pre-treatment. In contrast, SnPP had no significant effect on CFR in control or haemin-treated hearts, suggesting that endogenous HO-2 may not be involved in the control of CFR. In addition, haemin pre-treatment did not significantly affect contractility compared with control (6.6 ± 0.6g and 6.5 ± 0.1g respectively) (Figure 4.1.3B). Thus, induction of HO-1 expression did not appear to influence the force of contraction in the pre-ischaemic rat heart perfused at constant-pressure. In contrast, SnPP treatment significantly reduced the force of contraction regardless of control or haemin pre-treatment (3.8 ± 0.1g and 3.9 ± 0.4g respectively). Thus, this effect appears to be limited to SnPP treatment and may occur as a result of a non-specific action. In contrast to its lack of effect on LVDT, haemin pre-treatment significantly reduced heart rate from 240.4 BPM in control hearts to 196.4 BPM ($p<0.05$, Figure 4.1.4A). SnPP did not significantly affect heart rate in rat hearts treated with saline or haemin (260 ± 39 BPM and 210 ± 24 BPM respectively). This result parallels the effect on CFR, implicating a possible connection between the two events. For example, heart rate may be reduced as a result of the vasoconstrictor effect of haemin treatment. Haemin and SnPP-alone or in combination did not affect basal tension immediately prior to the start of ischaemia (Figure 4.1.4B).

(A) Heart Rate



(B) Basal Tension

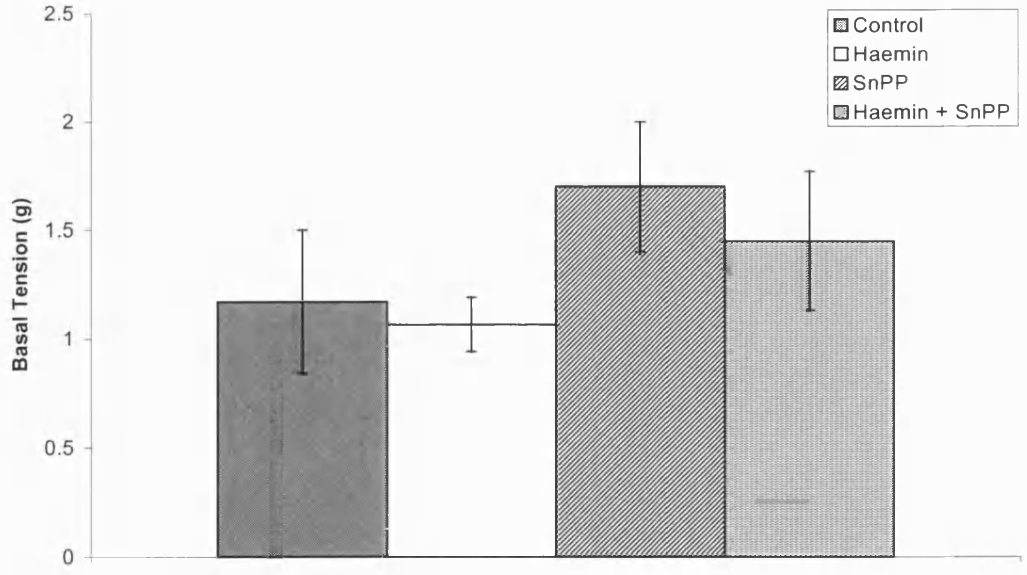


Figure 4.1.4

The effect of haemin and SnPP pre-treatment alone and in combination on heart rate (A) and basal tension (B) in the pre-ischaemic rat heart perfused at a constant-pressure of 70 mmHg.

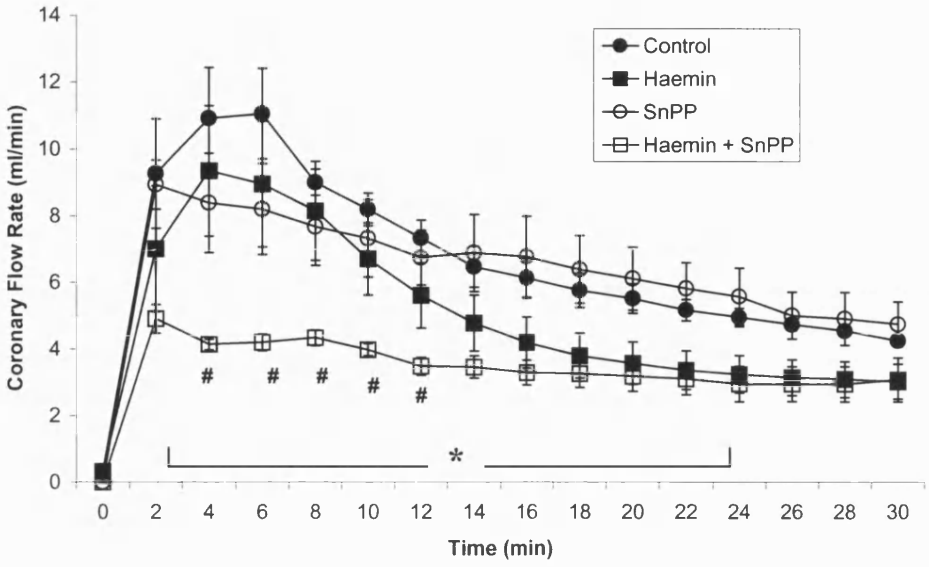
Rats were pre-treated with saline, 75 μmol/kg haemin (24hour), 40 μmol/kg SnPP (1 hour) and haemin + SnPP. Values are expressed as mean ± SEM. * where $p<0.05$ compared with control (n=5).

4.1.3. The effect of haemin pre-treatment on the recovery of heart perfused at a constant pressure of 70 mmHg following 20 min ischaemia / 30 min reperfusion.

Haemin pre-treatment did not significantly affect CFR during reperfusion, compared with control (Figure 4.1.5A). The presence of SnPP in haemin-treated hearts brought about a significant reduction in CFR (t=4-12 min) compared with haemin alone, and also compared with control and SnPP (t=2-24 min). Therefore, haemin and SnPP appear to blunt reactive hyperemia. A potential additive effect of haemin + SnPP may be involved in the significant reduction of CFR.

In contrast to the situation in constant-flow-perfused hearts, the recovery of contractility in the constant-pressure-perfused rat heart was not significantly increased in haemin-treated hearts (Figure 4.1.5B). This may be due to the high level of recovery observed in control hearts (82.7 ± 5% compared with 73.5 ± 19% in haemin-treated hearts). Recovery from I/R in hearts perfused at constant pressure is increased compared with data observed in the constant-flow perfusion model (Chapter 3), where control hearts recovered by 14 % and haemin-treated hearts recovered by 45 %, indicating that the constant-pressure model itself may confer protection after 20 min ischaemia. In addition, this result demonstrates that there is a very small therapeutic window in which to establish whether or not haemin can aid protection in this model. Furthermore, the recovery in contractility observed in control and haemin-treated hearts was significantly reduced in the presence of SnPP (21.8 ± 11 % and 29.3 ± 14 % respectively). SnPP also reduced contractility in the pre-ischaemic heart (Figure 4.1.2A). As haemin treatment exerts little influence on the contractility of the rat heart, it is likely that the reduction in recovery may be produced by a non-specific action from SnPP treatment. Alternatively, HO may be involved in the control and recovery of contractility, but an increase in HO-1 expression may not potentiate the effect.

(A) CFR during reperfusion



(B) Recovery of LVDT

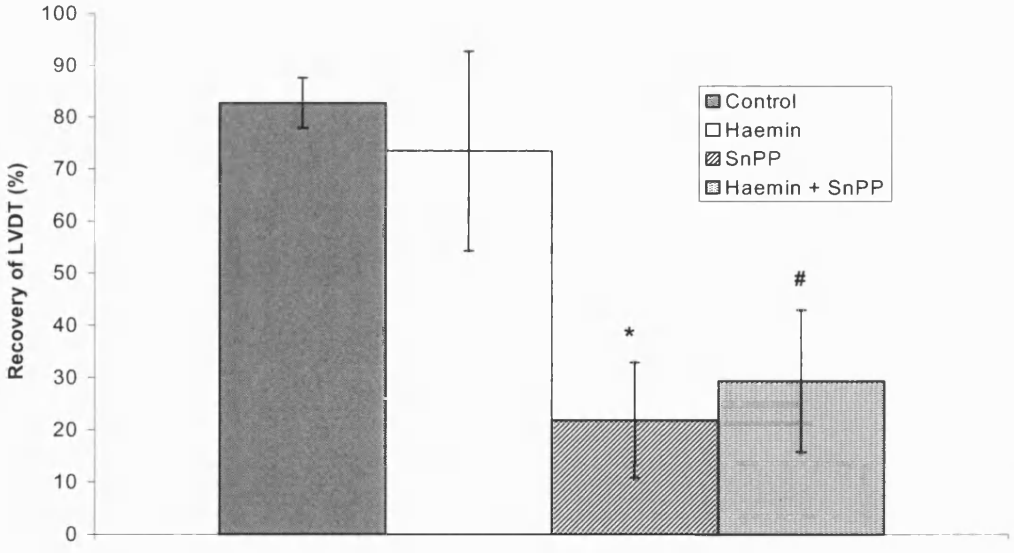
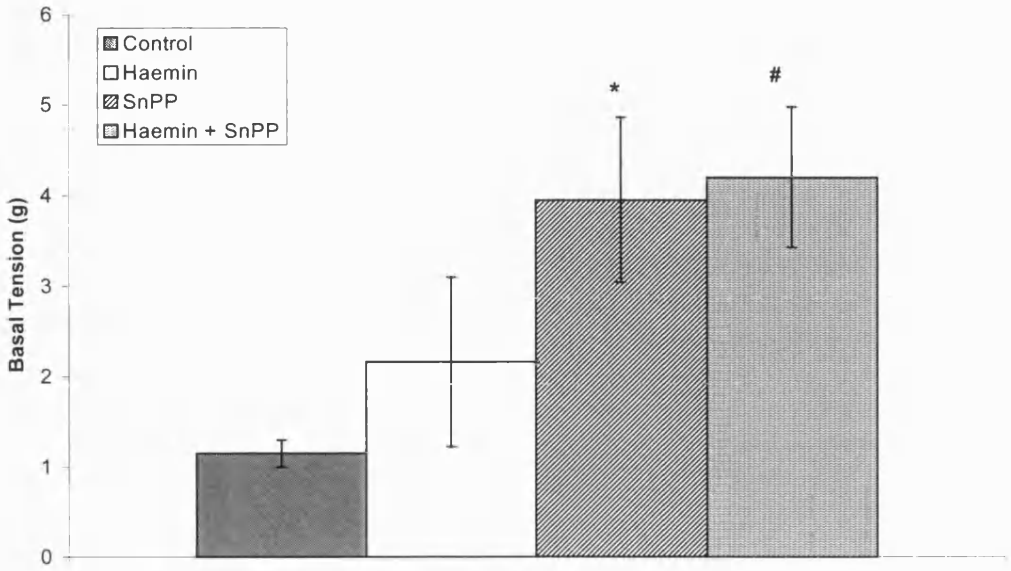


Figure 4.1.5

The effect of haemin and SnPP pre-treatment alone and in combination on CFR (A) and recovery of LVDT (B) in the post-ischaemic rat heart perfused at a constant pressure of 70 mmHg.

Rats were pre-treated with saline, 75 μmol/kg haemin (24hour), 40 μmol/kg SnPP (1 hour) and haemin + SnPP. Values are expressed as mean ± SEM. * where $p<0.05$ compared with control and SnPP (t=2-24min for CFR) or # $p<0.05$ compared with haemin, (n=5). Note: some error bars may fall within symbol size.

(A) Basal tension at the end of reperfusion



(B) Tissue bilirubin production

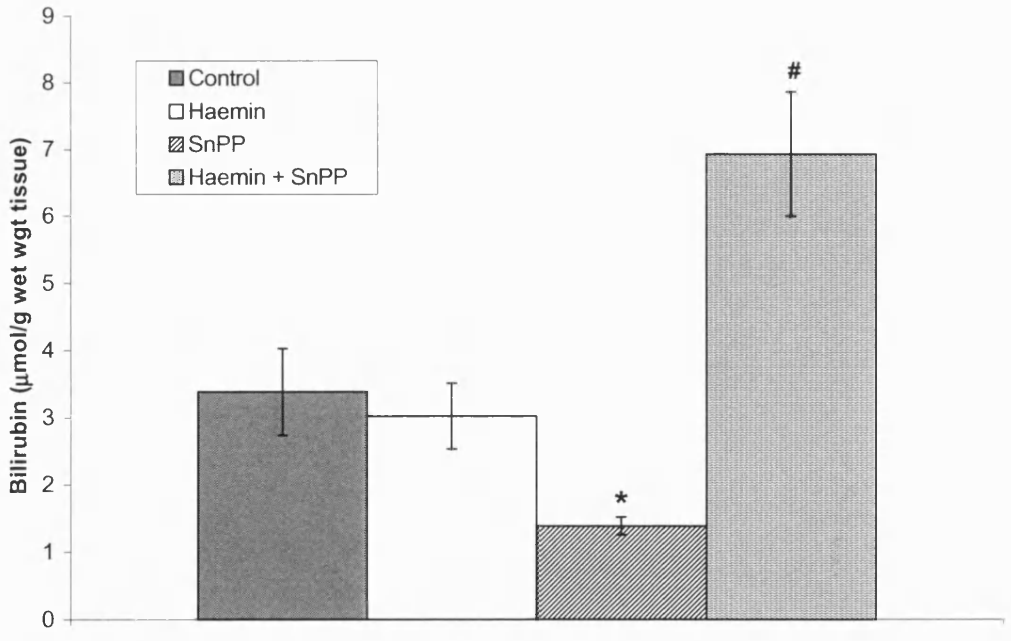


Figure 4.1.6

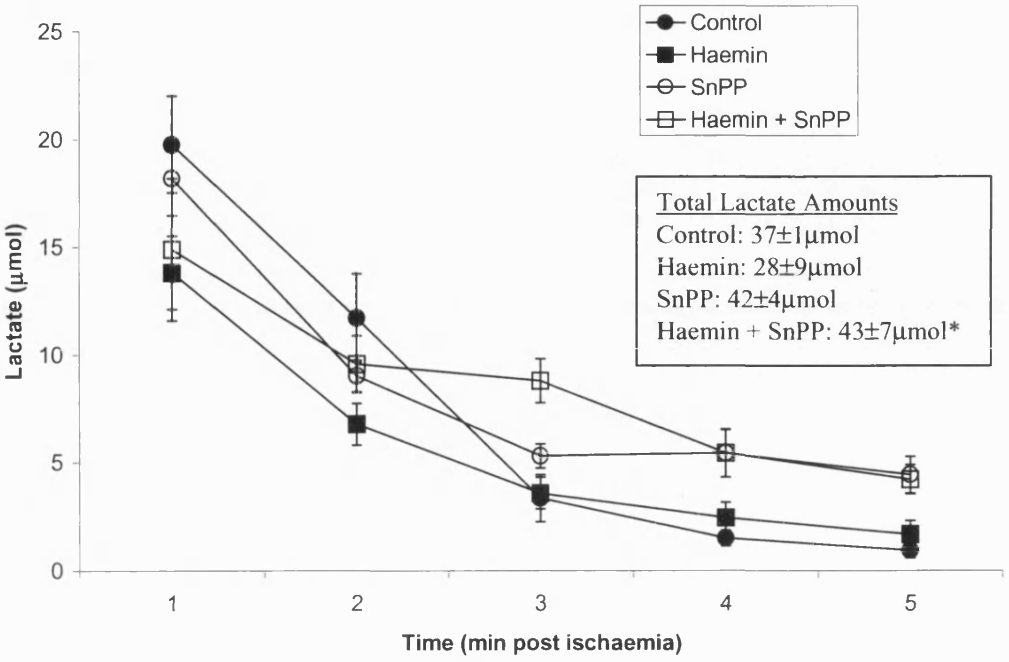
The effect of haemin and SnPP pre-treatment on basal tension (A) and tissue bilirubin levels (B) at the end of I/R in hearts perfused at a constant-pressure of 70 mmHg.

Rats were pre-treated with i.p. injections of saline, 75 µmol/kg haemin, and 40 µmol/kg SnPP (in the presence of haemin or alone). Values are expressed as mean ± SEM. * where $p<0.05$ compared with control, and # $p<0.05$ compared with haemin (n=5).

At the end of reperfusion, the basal tension observed in control and haemin-treated hearts did not differ significantly from each other (Figure 4.1.6A), and were also not different to the measurements taken at the pre-ischaemic period. In contrast, SnPP treatment in control and haemin-treated hearts caused a significant increase in basal tension at the end of the reperfusion period. After I/R, heart tissue was assayed for the quantity of tissue bilirubin. Haemin treatment did not significantly affect tissue bilirubin levels (3.0 ± 1 μmol bilirubin/g wet weight tissue) compared with control (3.4 ± 1 μmol bilirubin/g wet weight tissue) (Figure 4.1.6B). In the constant-flow model, the same dose of haemin increased bilirubin levels to approximately 6.5 μmol bilirubin/g wet weight tissue, whereas at constant-pressure, haemin pre-treatment induced the production of approximately 3 μmol bilirubin/g wet weight tissue. In addition, as a similar amount of bilirubin was detected in control tissues perfused at constant-flow and constant-pressure, it is likely that the protection observed under these conditions may be due to the method of perfusion itself rather than an increase in HO-1 activity or bilirubin production. The combination of haemin + SnPP significantly increased bilirubin production, which was surprising as SnPP actually reduced bilirubin production. Haemin and/or SnPP did not significantly affect the washout of lactate during the first 5 min of reperfusion, although there was a significant increase in total lactate release in hearts treated with haemin + SnPP compared with those treated with haemin alone (Figure 4.1.7A), whereas the combination of haemin + SnPP significantly increased total lactate release compared with haemin alone.

The release of nitrite in the pre- and post-ischaemic rat heart was measured to determine if the vasoconstrictor effect of haemin on CFR was due to an inhibitory effect on NO release. The results demonstrate that haemin has little effect on nitrite release in the pre-ischaemic heart compared with control (Figure 4.1.7B). On reperfusion, there is a significant decrease in nitrite release in haemin-treated hearts during the first minute of reperfusion compared with control. Interestingly, SnPP treatment significantly increased NO release in both the pre- and post-ischaemic heart ($p<0.05$ compared with control and haemin). This suggests that there may be further non-specific effects involving SnPP treatment on NO release.

(A) Lactate washout during first 5min of reperfusion



(B) Nitrite production in the pre- and post-ischaemic rat heart

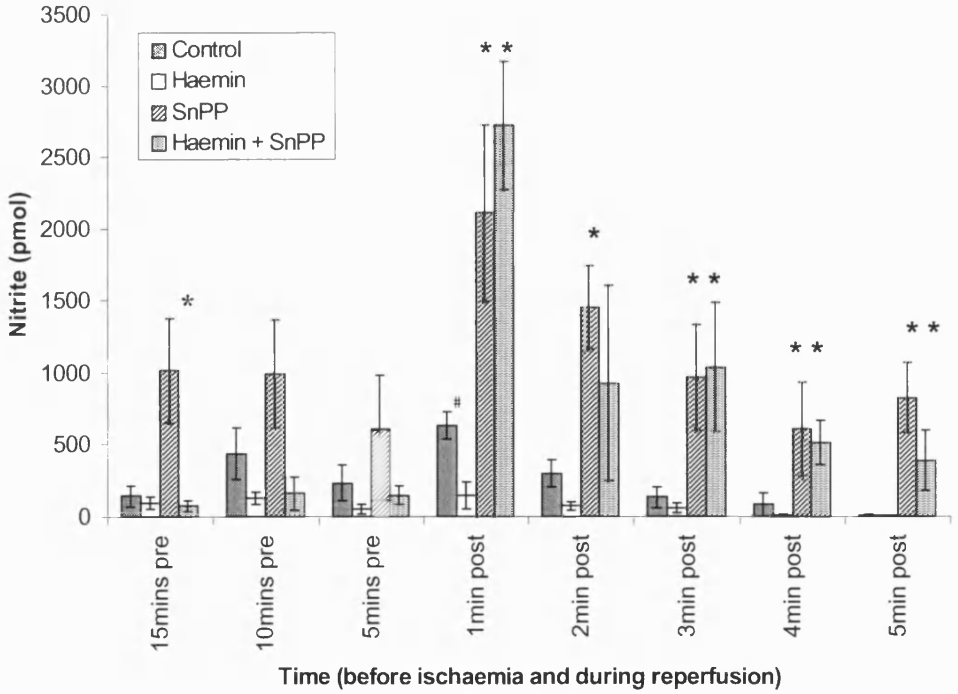


Figure 4.1.7

The effect of haemin and SnPP pre-treatment on nitrite release pre- and post- ischaemia in rat heart perfused at a constant-pressure of 70mmHg.

Rats were pre-treated with saline, 75 µmol/kg haemin (24hour), 75µmol/kg haemin and 40µmol/kg SnPP or 40 µmol/kg SnPP (1hour) alone. Values are expressed as mean ± SEM. * where $p<0.05$ compared with control and haemin and # compared with control (n=5). Note: some error bars fall within symbol size.

4.2 The effect of increasing the duration of the ischaemic period on the recovery of cardiac function in the constant-pressure-perfused rat heart

The following experiments were used to compare the effect of increasing ischaemic injury on the recovery of control hearts. The effect of haemin pre-treatment on the recovery of cardiac function after 30 min I/R was also determined. The data from these experiments helped to establish whether haemin's protective effect post I/R remained after a more severe and potentially irreversible ischaemic injury.

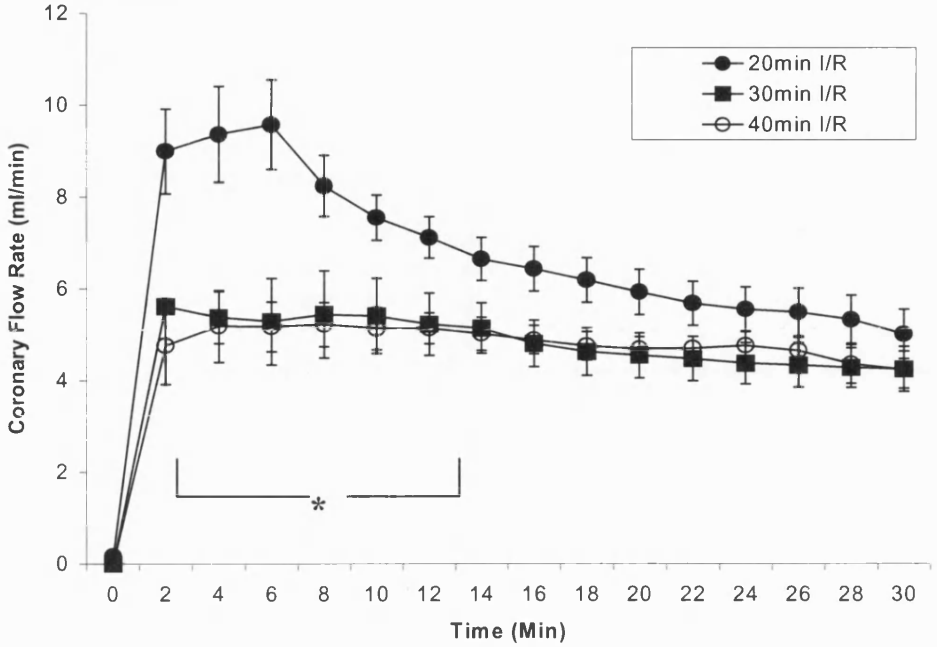
4.2.1 The effect of differing ischaemic insults on the recovery of control hearts perfused at 70 mmHg.

After an ischaemic period of increased duration (30-40 min), CFR was significantly reduced during the initial 12 min of reperfusion (Figure 4.2.1A). Therefore, the increase in duration of ischaemia is responsible for abolishing the reactive hyperaemia response to ischaemia. Consequently, it seems that there may be either a reduction in vascular responsiveness, or a decreased release of vasoactive mediators or disruption in the balance of vasoactive mediators during the first 10 min of reperfusion. Overall, this effect on reactive hyperemia is the most significant difference, as all 3 groups end with similar CFRs after 30 min reperfusion.

Recovery of contractility is also significantly reduced after increasing the duration of the ischaemic periods (Figure 4.2.1B). There is very little difference between the recovery of hearts after 30 and 40min ischaemia (3.4 ± 1 % and 3.9 ± 1 %, respectively). This suggests that the duration of the ischaemic period is vital in determining the recovery of contractility during reperfusion.

The reduction in recovery of contractility after 30 or 40 min ischaemia proceeded in parallel with an increase in basal tension (Figure 4.2.2A). There is a significant increase in basal tension after 30 and 40 min ischaemia. Increasing the duration of ischaemic injury to 40min caused a significant increase in bilirubin production (Figure 4.2.2B). HO-1 activity may increase in response to a longer ischaemic time course.

(A) CFR during reperfusion



(B) Recovery of LVDT during reperfusion

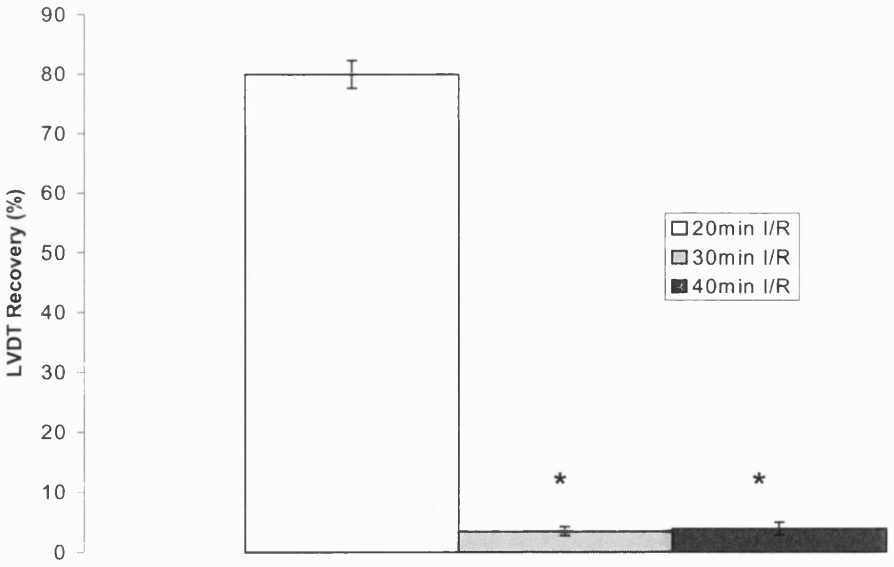
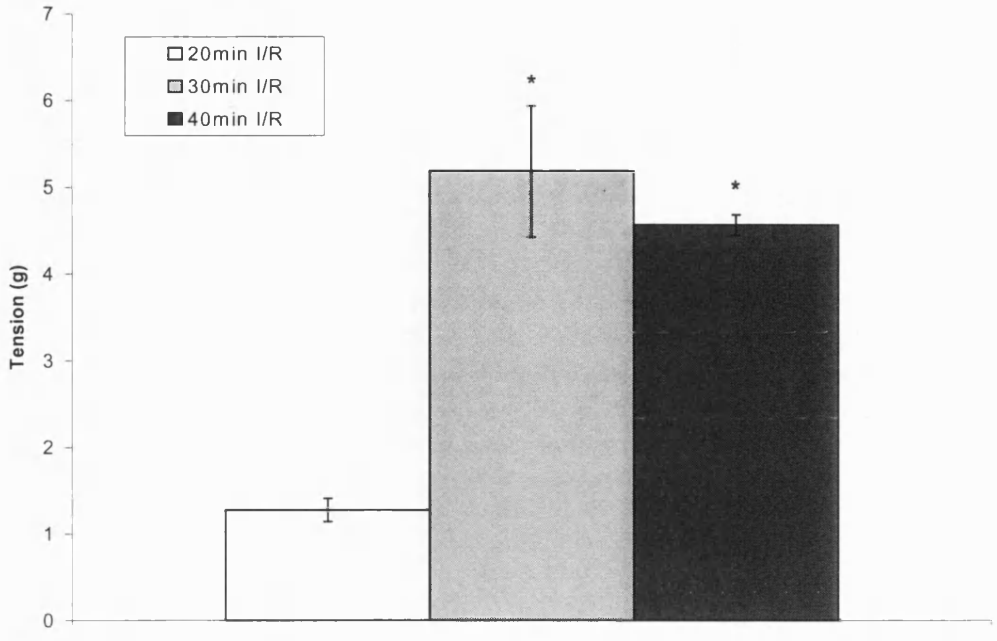


Figure 4.2.1

The effect of increasing ischaemic period on CFR (A) and recovery of LVDT (B) in the post-ischaemic control rat heart perfused at a constant-pressure of 70mmHg.

Rats were treated with saline (24hour) prior to heart removal. Values are expressed as mean ± SEM. * where $p<0.05$ compared with control heart after 20 min ischaemia (t=2-12 min post ischaemia for CFR), and n=4-5. Note: some error bars fall within the size of the symbol.

(A) Basal tension during reperfusion



(B) Tissue bilirubin production during reperfusion

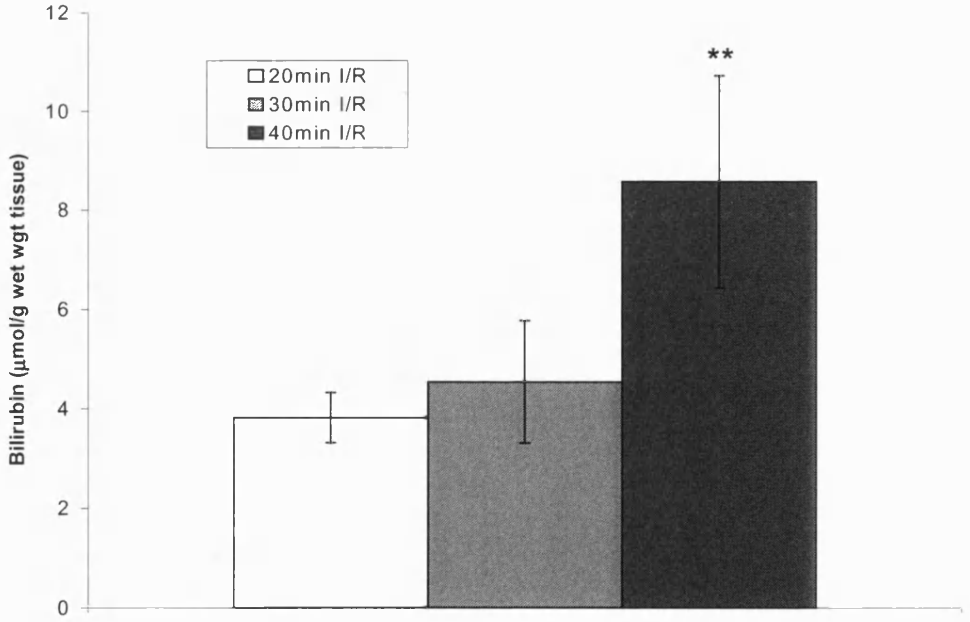


Figure 4.2.2

The effect of increasing ischaemic insult on basal tension (A) and tissue bilirubin levels (B) in post-ischaemic control rat hearts perfused at a constant-pressure of 70mmHg.

Rats were treated with saline (24hour) prior to removal of heart. Values are expressed as mean ± SEM, * where $p<0.05$ compared with control hearts exposed to 20 min I/R only or ** $p<0.05$ compared to 20 and 30 min I/R (n=4-5).

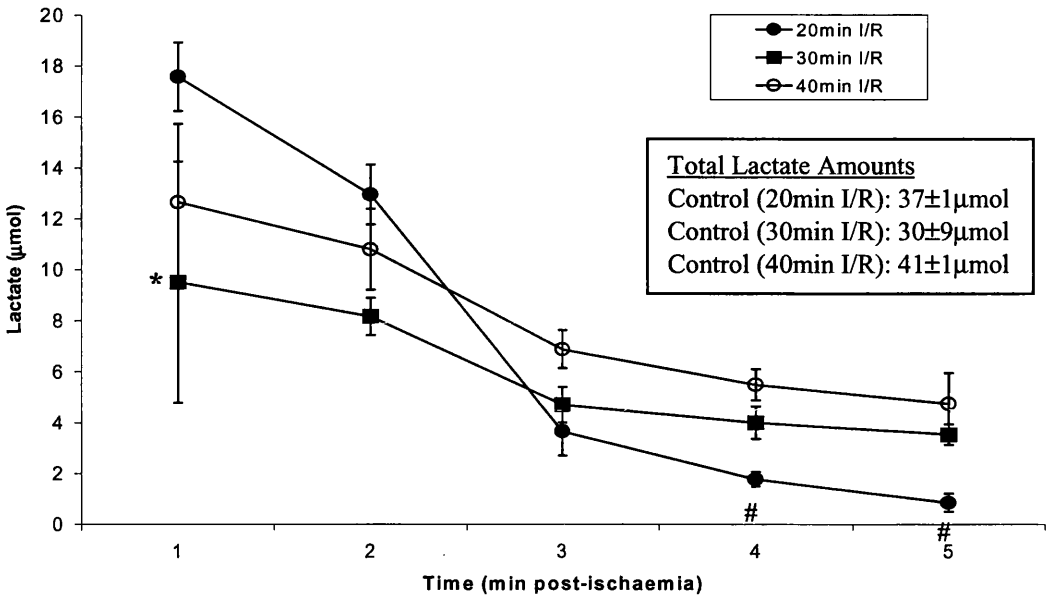


Figure 4.2.3.
The effect of increasing ischaemic insult on lactate washout in the post-ischaemic control rat heart perfused at a constant-pressure of 70mmHg.
Rats were pre-treated with saline (24hour). Values are expressed as mean ± SEM. * where $p<0.05$ 30 min ischaemia compared with 20 min ischaemia and # $p<0.05$ 40 min ischaemia compared with 20 min ischaemia, (n=5). Note: some error bars fall within the size of the symbol.

Increasing the duration of the ischaemic period seems to produce a more gradual lactate washout compared to the more rapid release seen after 20 min ischaemia (Figure 4.2.3), although total lactate release was not significantly affected by increasing the ischaemic insult. This response could be due to the reduction in reactive hyperaemia observed on CFR during the first 10 min + after reperfusion.

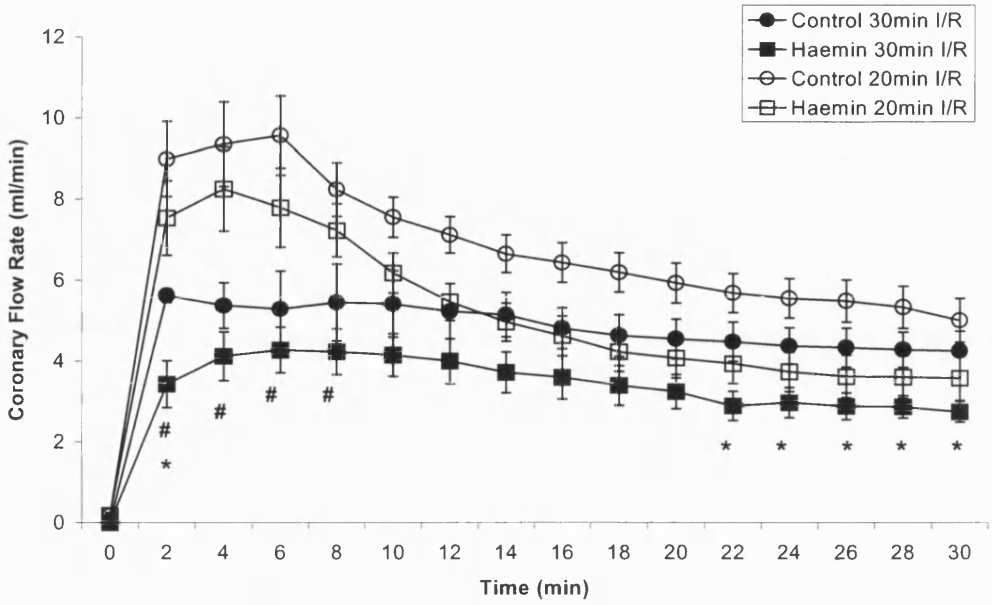
4.2.2 The effect of haemin on the recovery of cardiac function following 30min of ischaemia at a constant pressure of 70mmHg.

During reperfusion, CFR is significantly reduced in haemin-treated hearts subjected to 30 min I/R compared with control ($p<0.05$, $t=2$, 22-30) (Figure 4.2.4A). This is in contrast to the data observed after 20 min ischaemia, where haemin and control hearts showed a similar pattern of CFR after ischaemia. In addition, the reactive hyperaemia response to ischaemia is also abolished in both control and haemin-treated hearts as compared with CFRs recorded during reperfusion after a 20min ischaemic insult (see figure 4.1.4A).

The recovery of cardiac function after I/R was significantly reduced as the ischaemic insult was intensified (Figure 4.2.4B). After 30min I/R, control hearts recovered to 3.4 ± 1% compared to 3.3 % after haemin pre-treatment. This data suggests that haemin pre-treatment does not increase recovery of cardiac function after I/R compared with control. This is in agreement with the effect of haemin treatment on recovery from I/R after 20 min ischaemia. After 20 and 30 min ischaemia, the hearts recovered to a similar extent in control and haemin-treated hearts. Supplementary to the effect on recovery of cardiac function, 30 min I/R significantly increased basal tension in both haemin and control hearts compared with those subjected to 20 min ischaemia (Figure 4.2.5A). This is indicative of Ca^{2+} overload, and may indicate a reason for the significant reduction in the restoration of contractility during reperfusion.

After 30 min I/R bilirubin levels are significantly increased in haemin-treated hearts compared with control (Figure 4.2.5B). Furthermore, the amount of bilirubin measured was significantly higher than that seen in hearts subjected to 20 min I/R ($p<0.05$). This shows that as ischaemic injury increases, the levels of bilirubin increase in haemin-treated hearts.

(A) CFR during reperfusion



(B) Recovery of LVDT

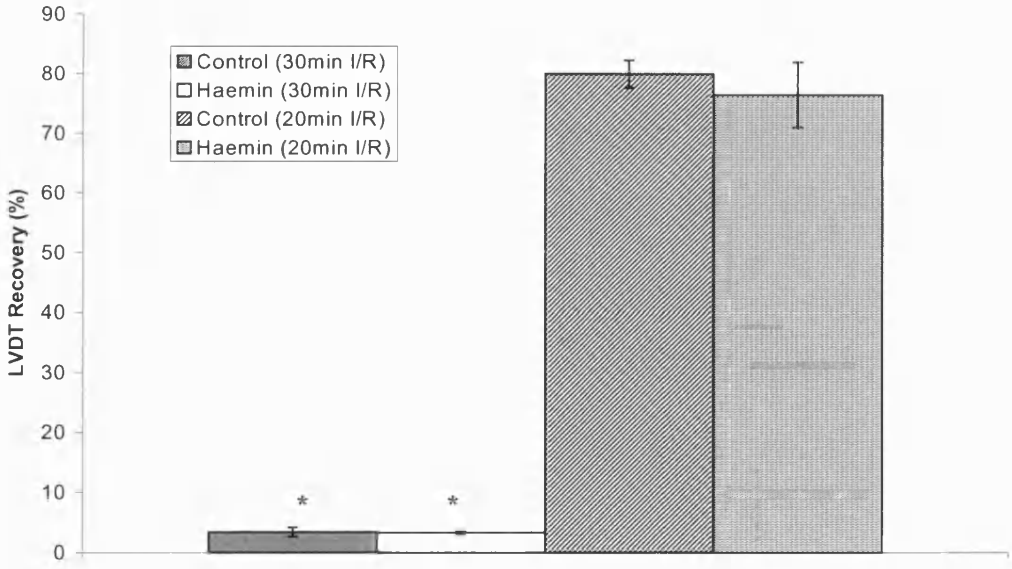
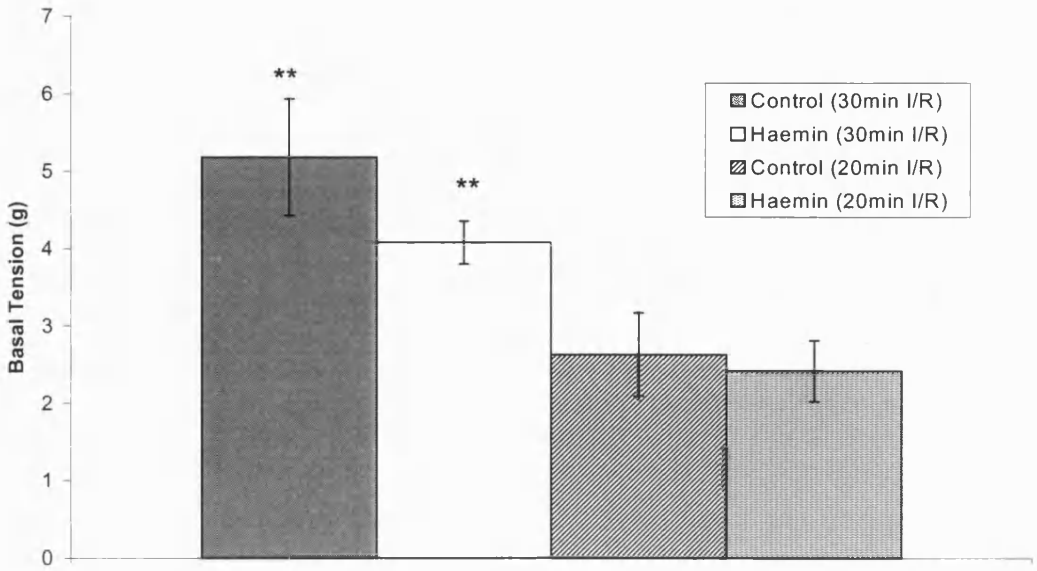


Figure 4.2.4

The effect of haemin on CFR (A) and recovery of LVDT (B) after 30min global ischaemia in the post-ischaemic haemin-treated rat heart perfused at a constant-pressure of 70mmHg.

Rats were treated with saline and 75 μmol/kg haemin (24hour) prior to heart removal. Values are expressed as mean ± SEM. * where $p<0.05$ compared with control after 30 min ischaemia (t=2, 22-30 min post ischaemia for CFR) or # where $p<0.05$ compared with control and haemin after 20 min ischaemia (n=5). Note: some error bars fall within the size of the symbol.

(A) Basal tension during reperfusion



(B) Tissue bilirubin production

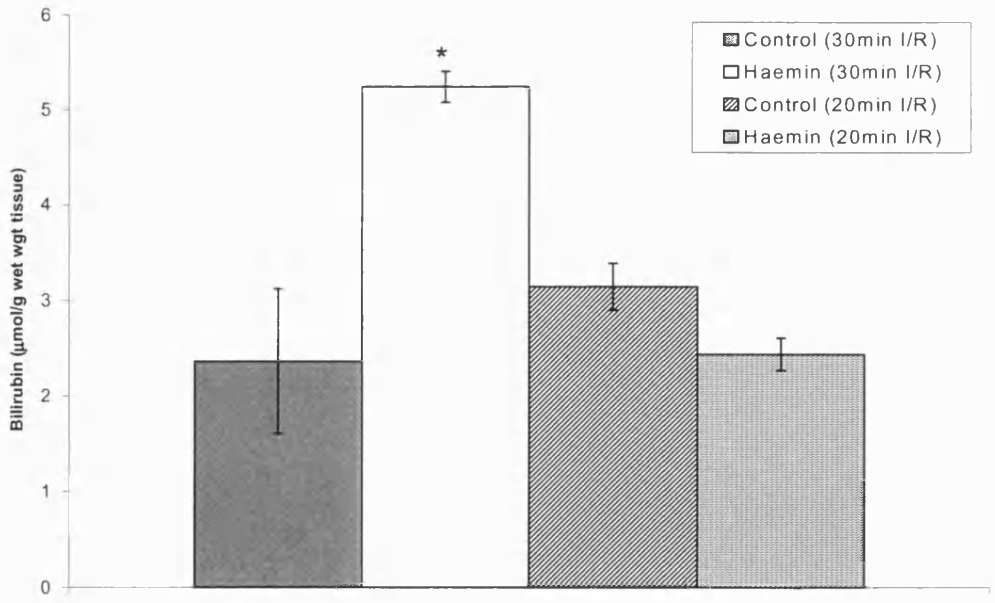


Figure 4.2.5

The effect of haemin on basal tension (A) and tissue bilirubin levels (B) in the post-I/R in hearts perfused at a constant pressure of 70 mmHg following 20 and 30 min ischaemia.

Rats were pre-treated with i.p. injections of saline and 75 µmol/kg haemin (24hour). Values are expressed as mean ± SEM. * where $p<0.05$ compared with control and ** $p<0.05$ compared with control and haemin post 20 min I/R, (n=5).

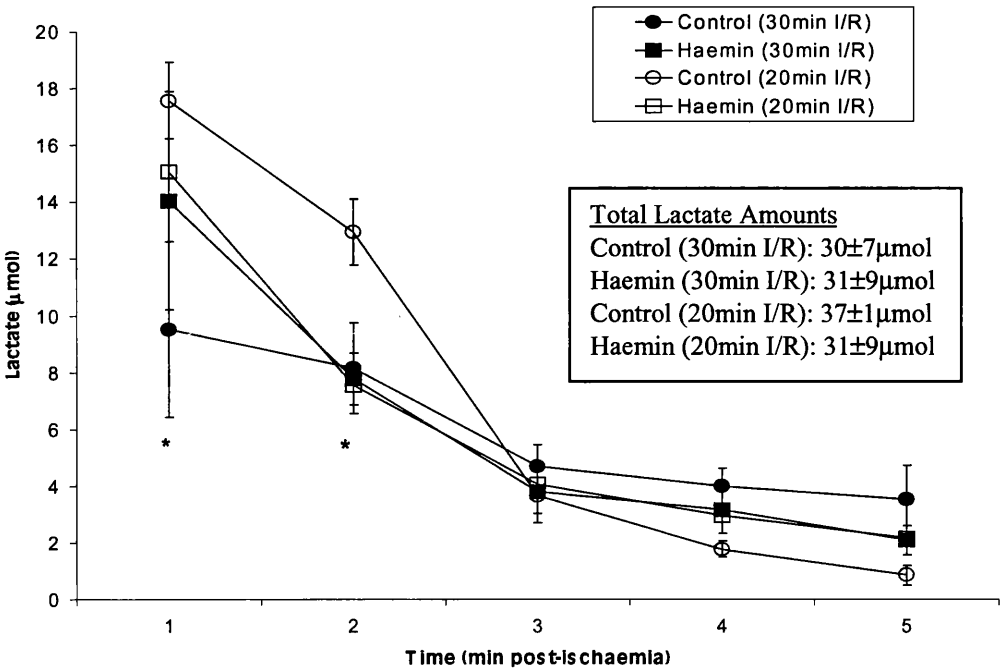


Figure 4.2.6

The effect of haemin on lactate washout in the first 5 min of reperfusion after 20 and 30min ischaemia in the rat heart perfused at a constant-pressure of 70mmHg.

Rats were pre-treated with saline or 75 µmol/kg (24hour). Values are expressed as mean ± SEM, * where $p<0.05$ compared with control (20 min Ischaemia) (n=5).

Increasing the duration of the ischaemic period had very little effect on lactate washout or total lactate release during the first 5 min of reperfusion (Figure 4.2.6). The only significant effect was demonstrated during the first 2 min of reperfusion, where lactate washout was decreased in control hearts after 30 min ischaemia compared with control hearts exposed to 20 min ischaemia ($p<0.05$).

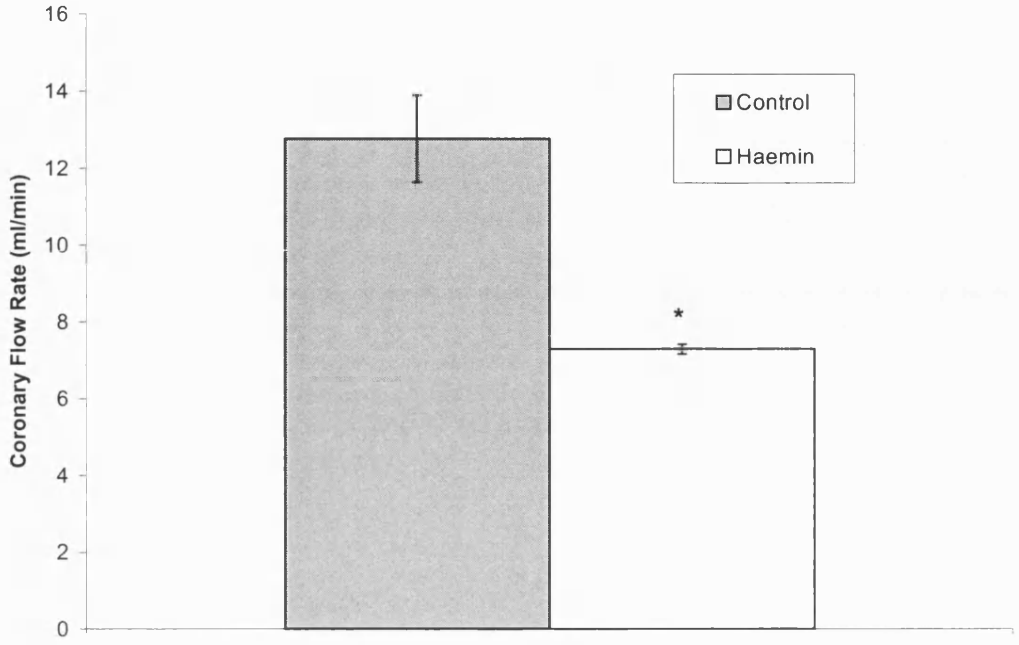
4.3 The effect of perfusion at a constant pressure of 130 mmHg on the action of haemin in the pre- and post- ischaemic rat heart.

The effect of haemin pre-treatment on the recovery of cardiac function in hearts perfused at a constant pressure of 130 mmHg was investigated to determine if the pressure of the perfusion system influenced the activity of haemin treatment. In the constant flow perfused heart, during the initial perfusion period the CPP of control hearts reached around 130-140 mmHg and 70-90 mmHg in haemin-treated hearts. The following experiments determined if a different initial pressure affected the recovery from I/R compared with a constant-pressure of 70mmHg as used in the initial experiments.

4.3.1. The effect of perfusion at a constant pressure of 130 mmHg in the pre-ischaemic rat heart.

The CFR of haemin-treated hearts perfused at 130 mmHg (7.3 ± 0.1 ml/min) is significantly lower than that of control hearts (12.8 ± 1.1 ml/min) (Figure 4.3.1A). Therefore, the vasoconstrictor effect of haemin is not dependent upon the pressure at which the tissue is perfused. The CFRs in hearts perfused at 130 mmHg increase to almost double the CFRs measured at 70 mmHg in both control and haemin-treated hearts (Figure 4.1.1A). Haemin treated hearts perfused at a constant pressure of 130 mmHg did not display any significant difference in contractility compared with control (Figure 4.3.1B). This suggests that the influence of HO-1 on the CFR does not affect the force of contraction, i.e. the vasoconstrictor effect of haemin does not induce a change in contractility. This may be due to the method of perfusion, as the constant-pressure perfusion system allows the flow rate to change and thus to accommodate any metabolic or vascular changes in the model. The LVDT of hearts perfused at a constant-pressure of 130 mmHg was markedly lower than those previously recorded at 70 mmHg (Figure 4.1.1B). At a perfusion pressure of 130 mmHg, haemin pre-treatment had no significant effect on HR. Haemin pre-treatment increased heart rate after perfusion at 130 mmHg compared with haemin-treated hearts perfused at 70 mmHg (see Figure 4.1.2A). In the pre-ischaemic rat heart, there was no significant difference in basal tension in control and haemin-treated hearts perfused at 130 mmHg (Figure 4.3.2B).

(A) CFR – pre-ischaemia



(B) LVDT – pre-ischaemia

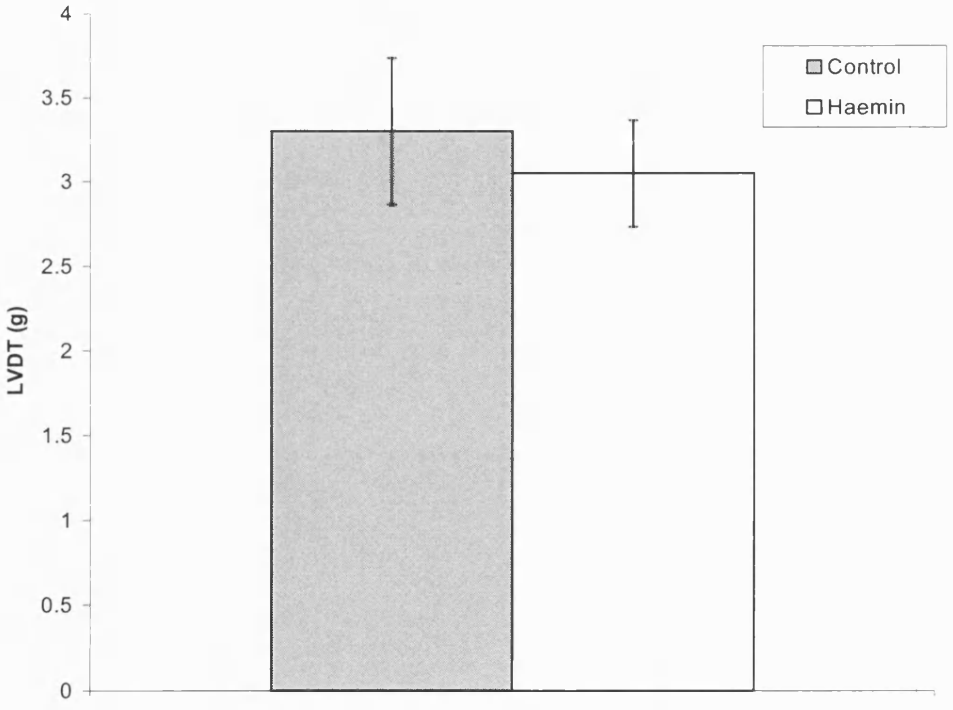
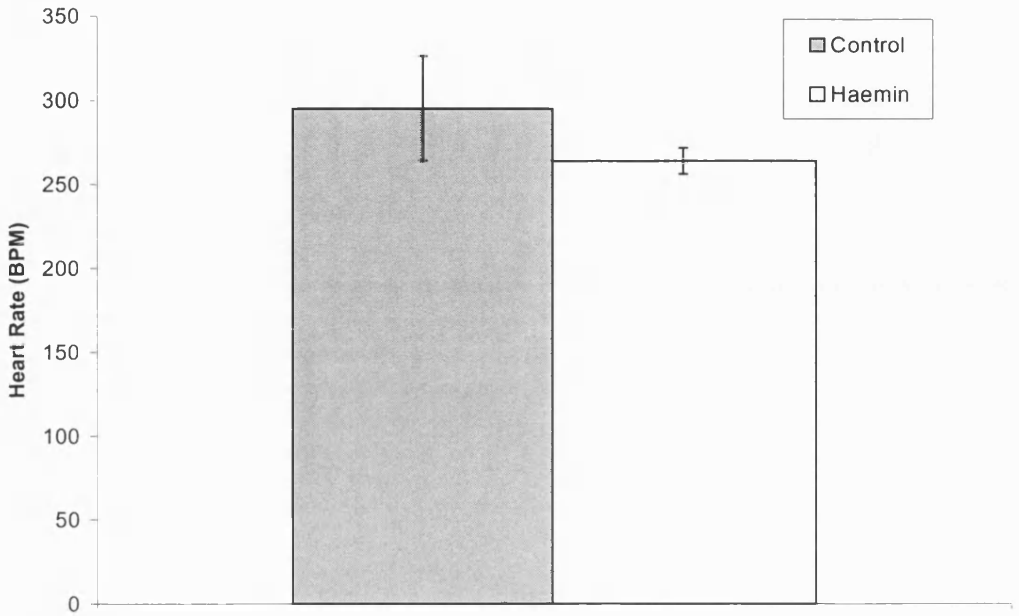


Figure 4.3.1

The effect of haemin pre-treatment on CFR (A) and LVDT (B) in the pre-ischaemic rat heart perfused at a constant pressure of 130 mmHg.

Rats were pre-treated with saline or 75 μmol/kg haemin (24hour). Values are expressed as mean ± SEM, * where $p<0.05$ compared with control (n=5).

(A) Heart rate – pre-ischaemia



(B) Basal tension

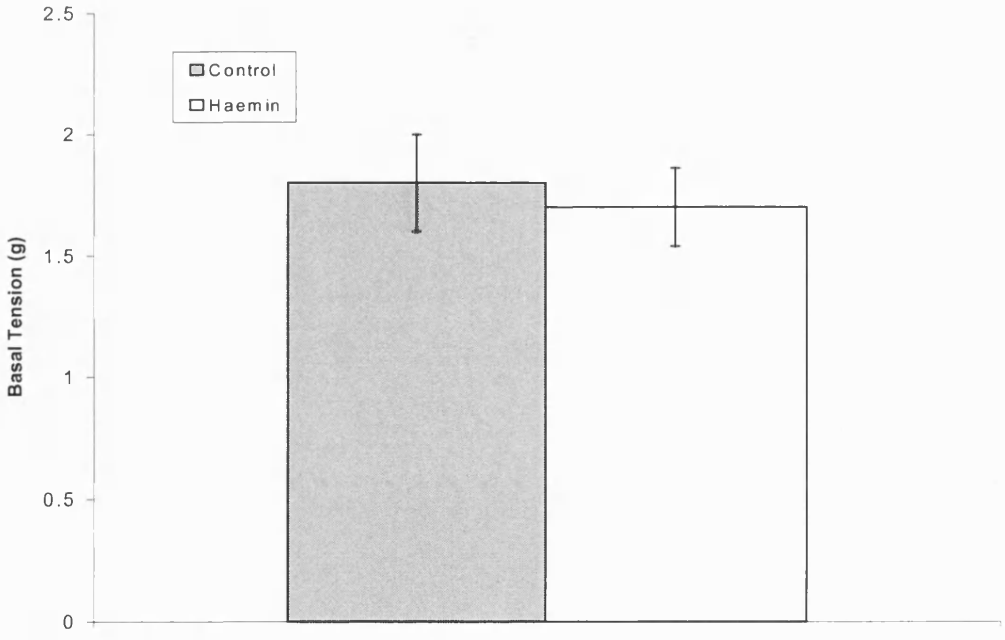


Figure 4.3.2

The effect of haemin pre-treatment on heart rate (A) and basal tension (B) in the pre-ischaemic rat heart perfused at a constant pressure of 130 mmHg.

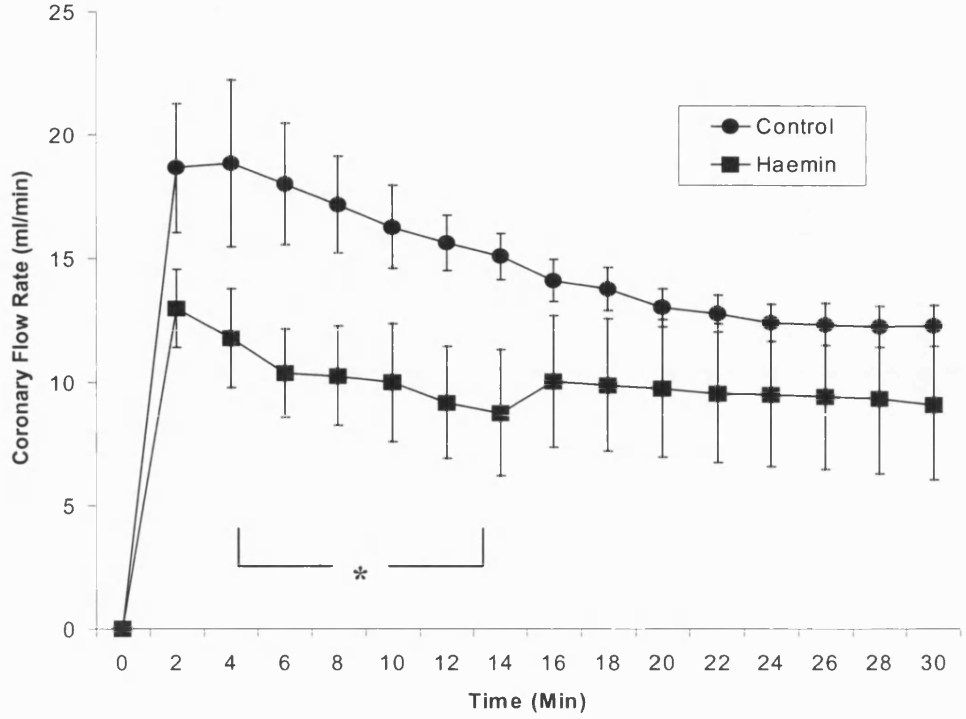
Rats were pre-treated with saline or 75 µmol/kg haemin (24hour). Values are expressed as mean ± SEM (n=5).

4.3.2 The effect of haemin pre-treatment on recovery from I/R in the rat heart perfused at a constant pressure of 130 mmHg.

During reperfusion, haemin pre-treatment reduced CFR after ischaemia compared to control (Figure 4.3.3A). The effect reached statistical significance when t=4-14 min. This indicates that the vasoconstrictor component of the response to haemin pre-treatment is not abolished by ischaemic insult in hearts perfused at 130 mmHg; this is in contrast to previous data recorded at 70 mmHg (Figure 4.1.3A), where the vasoconstrictor effect was not present during reperfusion. Therefore, the effect of haemin pre-treatment is more evident at a higher perfusion pressure. This suggests that the basal tone of the heart may affect the vascular effect of haemin after I/R. In addition, the CFR is markedly increased in control and haemin-treated hearts perfused at 130 mmHg compared with hearts perfused at 70 mmHg (Figure 4.1.3A). This is in agreement with the pre-ischaemic data where there is no evidence of autoregulation.

Haemin pre-treatment significantly increased recovery of contractility compared with control (Figure 4.3.3B) after I/R in hearts perfused at a constant pressure of 130 mmHg. This is in contrast to previously recorded data at 70 mmHg, where haemin pre-treatment did not confer any protection from I/R compared to control. In addition, the overall recovery in both control and haemin-treated hearts perfused at 130 mmHg was markedly lower than both treatment groups perfused at 70 mmHg (Figure 4.3.3B). Therefore, these data demonstrate that haemin exerts a greater protective effect at a higher pressure, and is more evident when the recovery of control hearts is reduced. In combination with figure 4.2.2, this indicates that in the constant-pressure-perfused rat heart, the level of protection conferred by HO-1 induction is dependent upon the basal pressure.

(A) CFR during reperfusion



(B) Recovery of LVDT during reperfusion

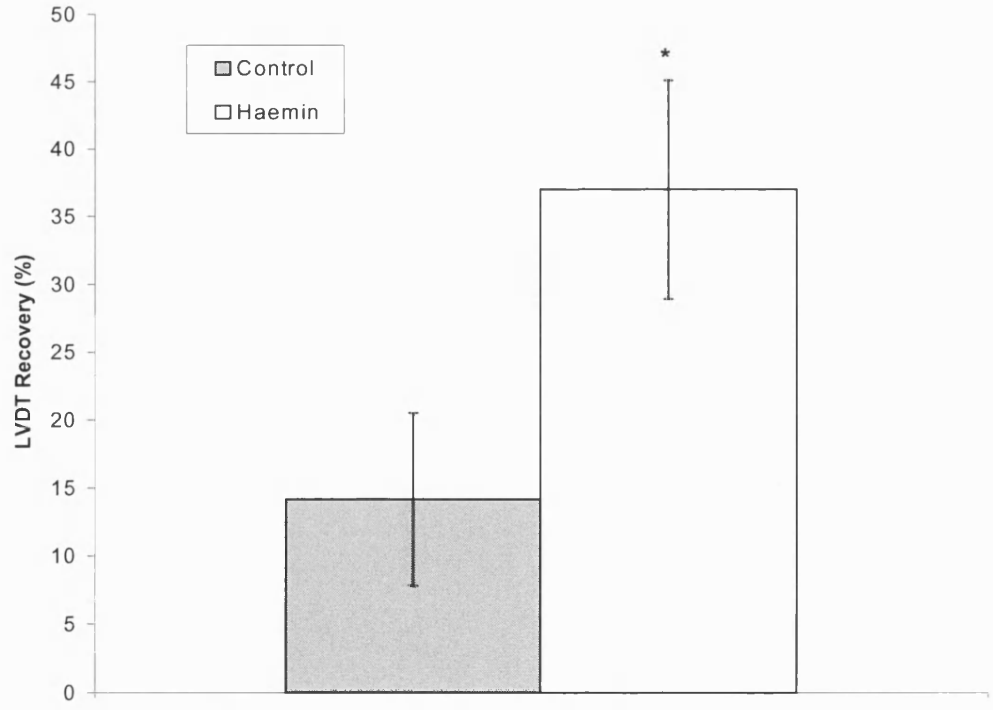
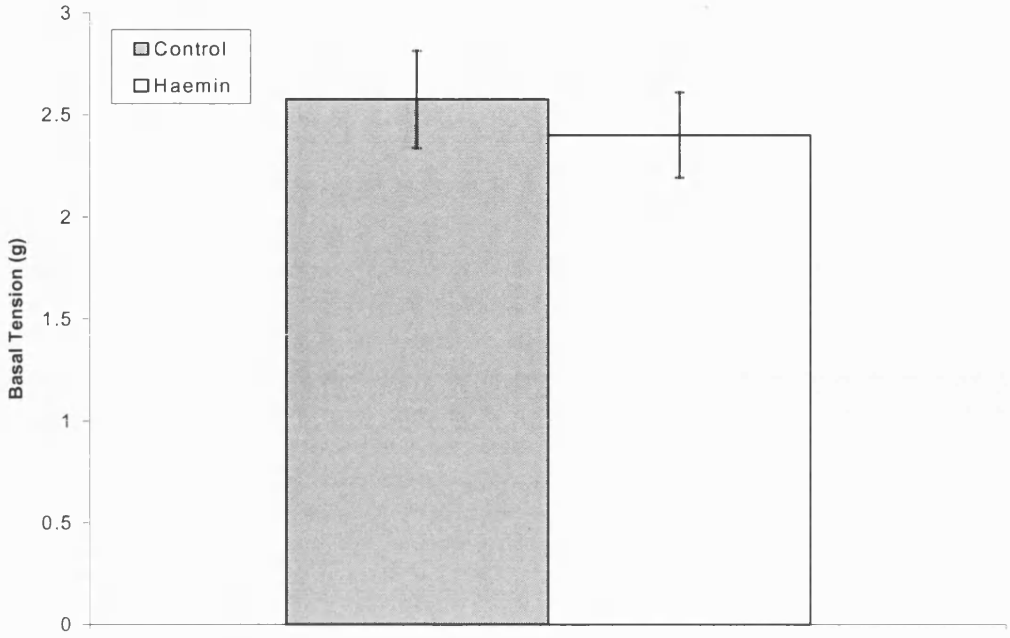


Figure 4.3.3
The effect of haemin pre-treatment on CFR (A) and recovery of LVDT (B) in the post-ischaemic rat heart perfused at a constant pressure of 130 mmHg.
Rats were pre-treated with saline or 75 μmol/kg haemin (24hour). Values are expressed as mean ± SEM. * where $p<0.05$ (t=4-14 min for CFR) compared with control (n=5).

A) Basal tension during reperfusion



(B) Tissue bilirubin production

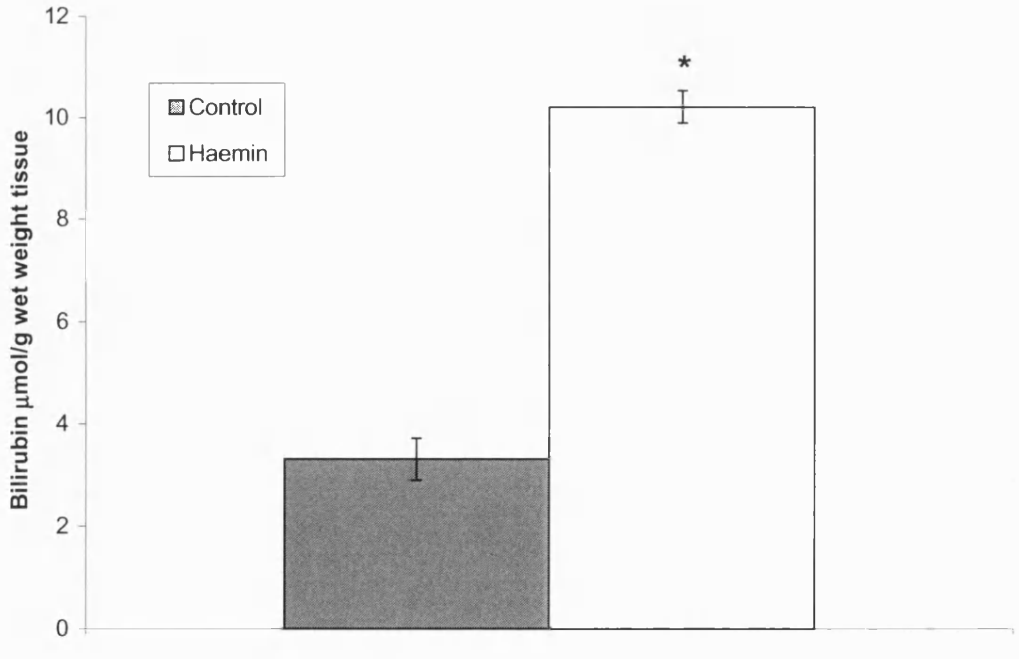


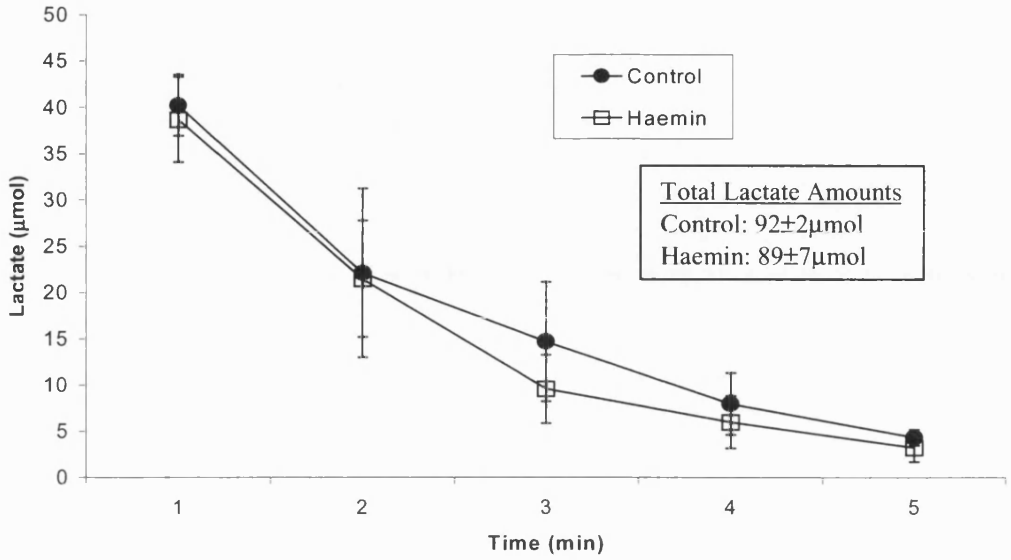
Figure 4.3.4

The effect of haemin pre-treatment on basal tension (A) and tissue bilirubin levels (B) after I/R in the rat heart perfused at a constant-pressure of 130 mmHg.

Rats were pre-treated with i.p. injections of saline or 75 µmol/kg haemin (24 hour). Values are expressed as mean ± SEM. * where $p<0.05$ compared with control (n=5).

The increased recovery of contractile function in haemin-treated hearts is accompanied by a relatively low basal tension after I/R (Figure 4.3.4A). After I/R, tissue bilirubin levels are significantly increased in haemin-treated hearts perfused at 130 mmHg (10.2 μmol bilirubin/g wet weight tissue) compared with control (3.3 μmol bilirubin/g wet weight tissue, $p<0.05$)(Figure 4.3.4B). Therefore, the improved recovery in cardiac function after I/R induced by haemin pre-treatment may be due to a significant increase in bilirubin levels, whereby the antioxidant nature of bilirubin aids the removal of ROS produced upon reperfusion. Furthermore, the levels of bilirubin are similar to those measured in haemin-treated hearts perfused at constant flow (Chapter 3), where a significant increase in recovery was also observed. In addition, lactate washout during the first 5 min of reperfusion was not significantly different in control and haemin-treated hearts perfused at 130 mmHg (Figure 4.3.5A). This suggests that the protective effect of haemin pre-treatment at 130 mmHg is not produced by an increase in anaerobic glycolysis, and/or by increased ATP production. There is no significant effect on nitrite release in haemin-treated hearts prior to ischaemic insult compared to control (Figure 4.3.5B). Therefore, these data indicate that the vasoconstrictor effect of haemin in the pre-ischaemic heart is unlikely to be produced by the inhibition of NO release. Haemin pre-treatment significantly reduces nitrite release in the post-ischaemic rat heart during the first 2 min of reperfusion ($p<0.05$). The previous data (Figure 4.3.3A) have indicated that haemin pre-treatment reduces the extent of reactive hyperaemia, which may be produced due to the prevention of NO synthesis or the removal of available NO. Furthermore, it is also possible that nitrite may be released exclusively in the first 5 min of reperfusion. Nitrite release is not increased in control hearts during reperfusion, suggesting that NO may not be responsible for the reactive hyperemia observed during reperfusion.

(A) Lactate washout during the first 5 min of reperfusion



(B) Nitrite release in the pre- and post-ischaemic heart.

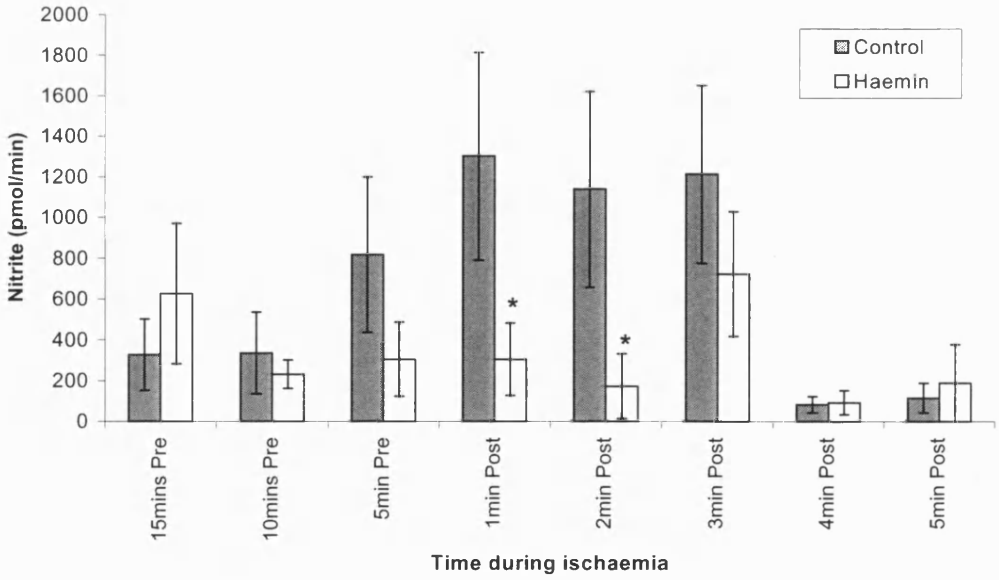


Figure 4.3.5

The effect of haemin pre-treatment on lactate washout in the post-ischaemic rat heart (A) and nitrite release in the pre- and post-ischaemic rat heart perfused at a constant pressure of 130 mmHg.

Rats were pre-treated with saline or 75 µmol/kg haemin (24 hour). Values are expressed as mean ± SEM. * where $p<0.05$ compared with control (n=5). Note: some error bars may fall within the size of the symbol.

4.4 Investigation of the effect of the iron chelator desferrioxamine (DFO) on the effect of haemin on the recovery from I/R in the rat heart perfused at a constant-pressure of 70 mmHg

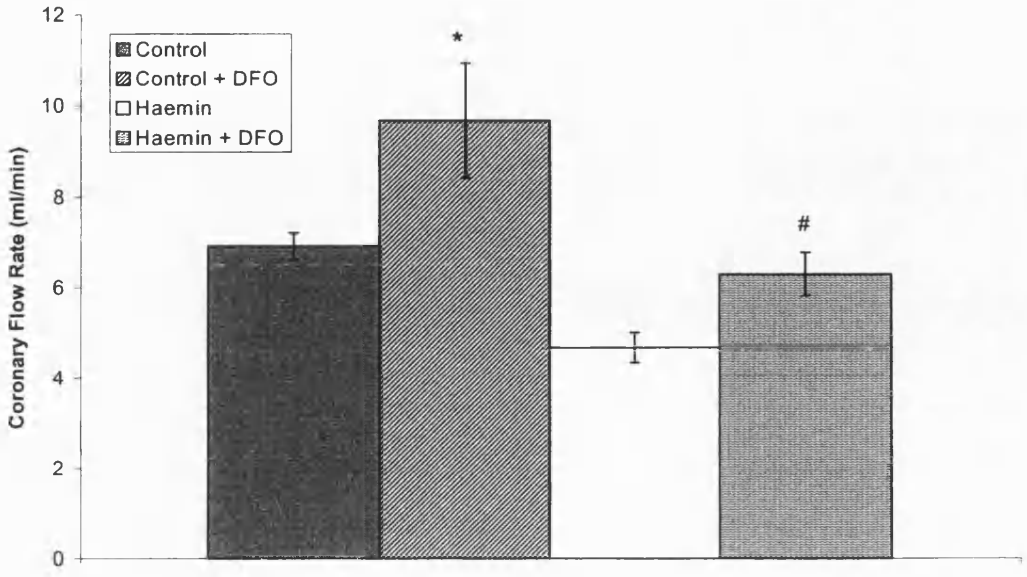
HO catalysis of haem produces bilirubin, CO and iron. Free iron release may have both protective and detrimental effects within cells. Therefore, desferrioxamine (DFO), an iron chelator, was employed in this study to investigate the possibility that haemin-induced iron release was modulating cardiac function.

4.4.1 The effect of desferrioxamine (DFO) perfusion in pre-ischaemic haemin-treated hearts.

DFO significantly increased CFR in both control and haemin-treated hearts (Figure 4.4.1A). These data suggest that free iron is exerting a vasoconstrictor effect in control and haemin-treated hearts. Alternatively, as there is an increase in the CFR in both control and haemin-treated tissues, it is possible that DFO is having an effect independent of haemin treatment.

In the presence of DFO, LVDT is significantly reduced in haemin-treated hearts (3.9 g) as compared with hearts treated with haemin alone (6.6 ± 1 g, $p<0.05$) (Figure 4.4.1B). This suggests that the presence of free iron significantly increased the force of contraction. This response is surprising as the converse might be expected as the presence of ROS, as a result of increased iron levels, has been shown to cause contractile dysfunction. The difference between DFO-treated and control samples was not statistically significant. This effect appears to be restricted to the force of contractility, as there is no significant difference on heart rate between treatment groups (Figure 4.4.2A). Therefore, the effect of DFO on contractility does not occur in response to a change in heart rate or vice versa. In addition, DFO did not significantly affect basal tension in control or haemin-treated hearts (Figure 4.4.2B).

(A) CFR – pre-ischaemia



(B) LVDT – pre-ischaemia

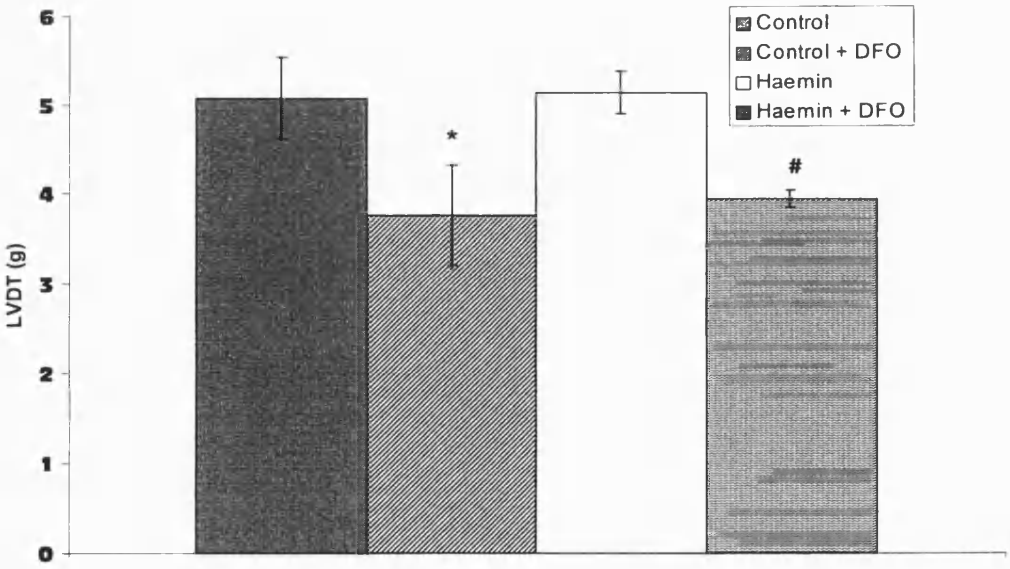
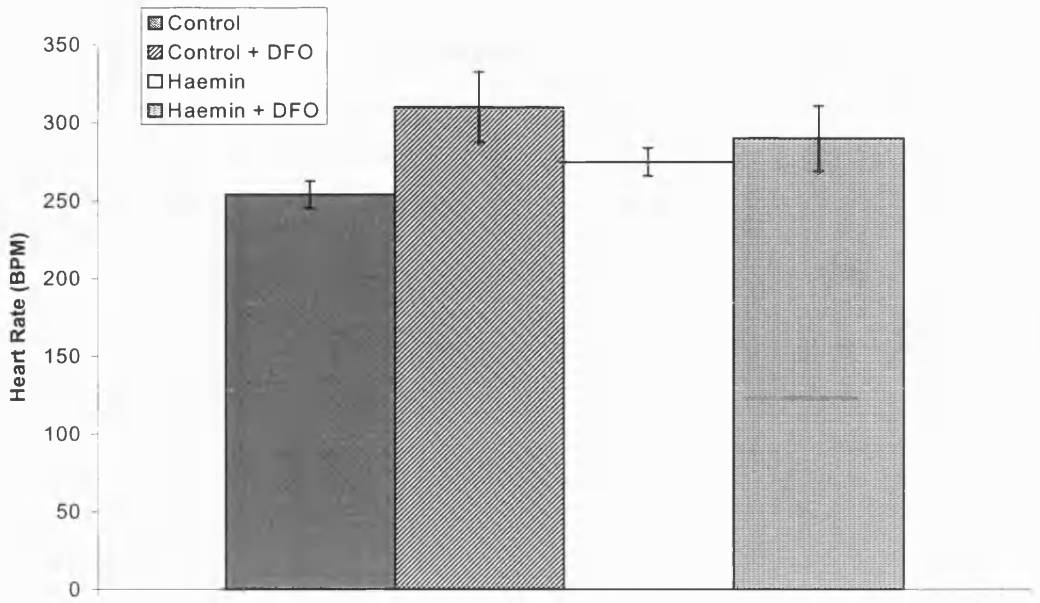


Figure 4.4.1.
The effect of DFO on CFR (A) and LVDT (B) in control and haemin-treated hearts prior to the onset of ischaemia.
Rats were pre-treated with saline or 75μmol/kg (24hour). Values are expressed as mean ± SEM. * $P<0.05$ compared with control and # where $p<0.05$ compared with haemin-treated hearts (n=5).

(A) Heart rate – pre-ischaemia



(B) Basal tension – pre-ischaemia

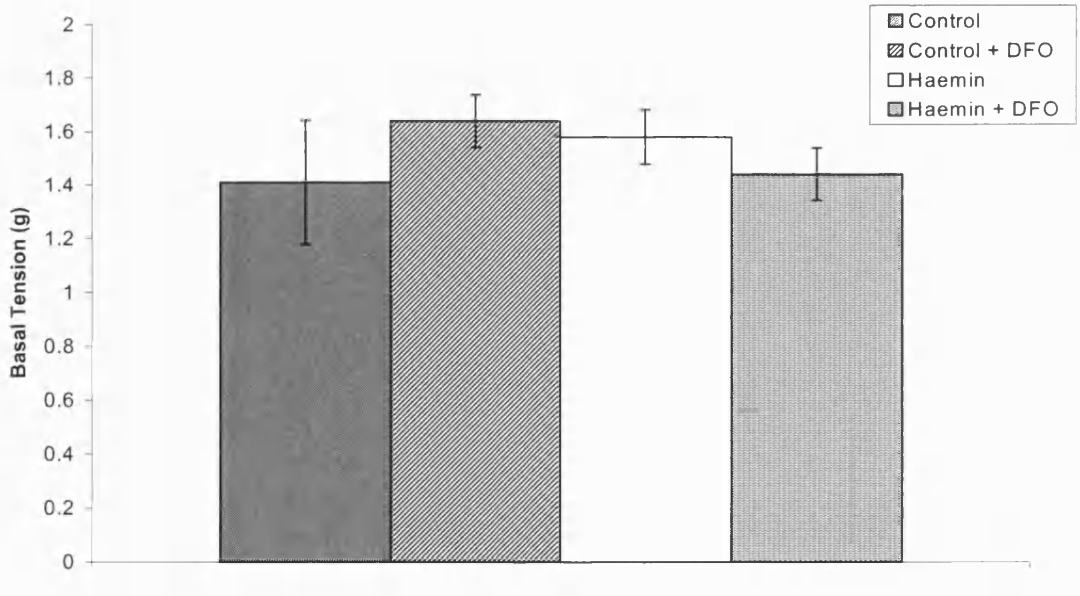


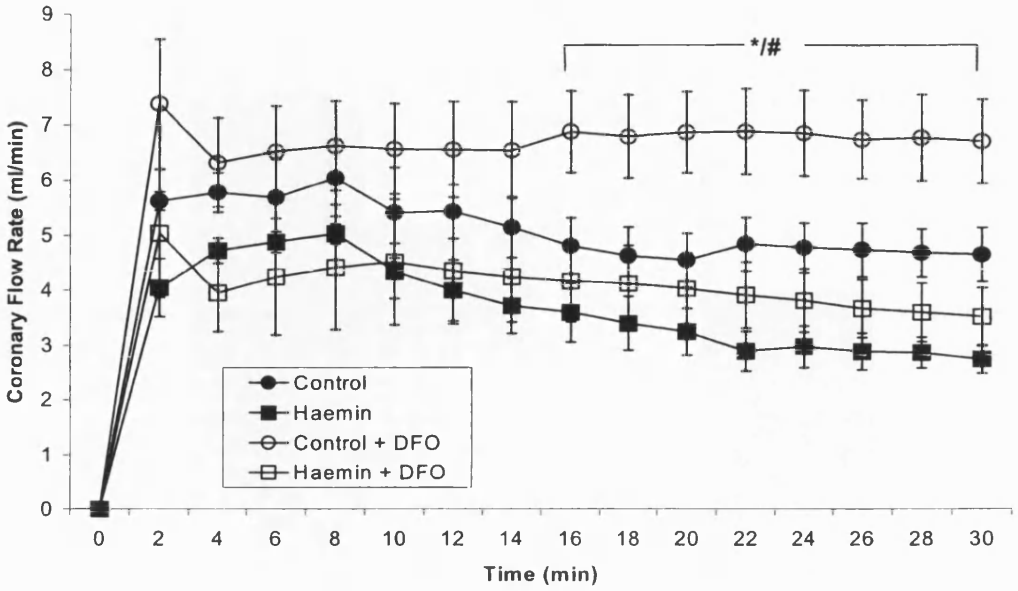
Figure 4.4.2.
The effect of DFO on heart rate (A) and basal tension (B) in control and haemin-treated pre-ischaemic rat hearts perfused at a constant-pressure of 70mmHg.
Rats were pre-treated with saline or 75 µmol/kg (24hour). Values are expressed as mean ± SEM, (n=5).

4.4.2 The effect of DFO on the recovery of cardiac function after 30 min global ischaemia and reperfusion in hearts perfused at a constant-pressure of 70 mmHg.

DFO significantly increased CFR in control hearts during reperfusion (t=18-30 min, $p<0.05$) (Figure 4.4.3B), suggesting that endogenous iron release has a vasoconstrictor effect under control conditions. In contrast, haemin-treated hearts exposed to DFO did not demonstrate any significant difference in CFR compared to haemin alone. These data indicate that free iron is not involved in the vasoconstrictor response of haemin-treated hearts to I/R. The use of DFO significantly increased recovery of cardiac function after 30 min I/R (Figure 4.4.3B, $p<0.05$) in both control and haemin-treated hearts. After 30 min I/R, recovery was minimal in control and haemin-treated hearts (3.4 ± 1 % and 3.3 % respectively), but on addition of DFO, recovery increased in both treatment groups (18.3 ± 4 % for control and 22.7 ± 7 % for haemin treatment). Therefore, the increase in recovery is associated with DFO alone, and is not affected by haemin pre-treatment. This suggests that the removal of free iron aids the recovery from I/R. Haemin in the presence of DFO significantly reduced basal tension compared with haemin alone (Figure 4.4.4A). There was no statistically significant reduction in the basal tension of control hearts perfused with DFO. This suggests that haemin + DFO prevent the ischaemia-induced increase in Ca^{2+} overload, as indicated by the basal tension.

Bilirubin production is significantly increased in haemin-treated hearts compared with control after 30 min I/R (Figure 4.4.4B, $p<0.05$). In contrast, haemin + DFO treatment significantly increased tissue bilirubin levels compared with haemin alone. Therefore, it is feasible that the increased bilirubin levels in haemin + DFO treated hearts could have contributed to the recovery of cardiac function from I/R, due to its antioxidant nature, although this does not explain the increased recovery in control hearts, suggesting that different mechanisms could be responsible.

(A) CFR – during reperfusion



(B) Recovery of LVDT after I/R

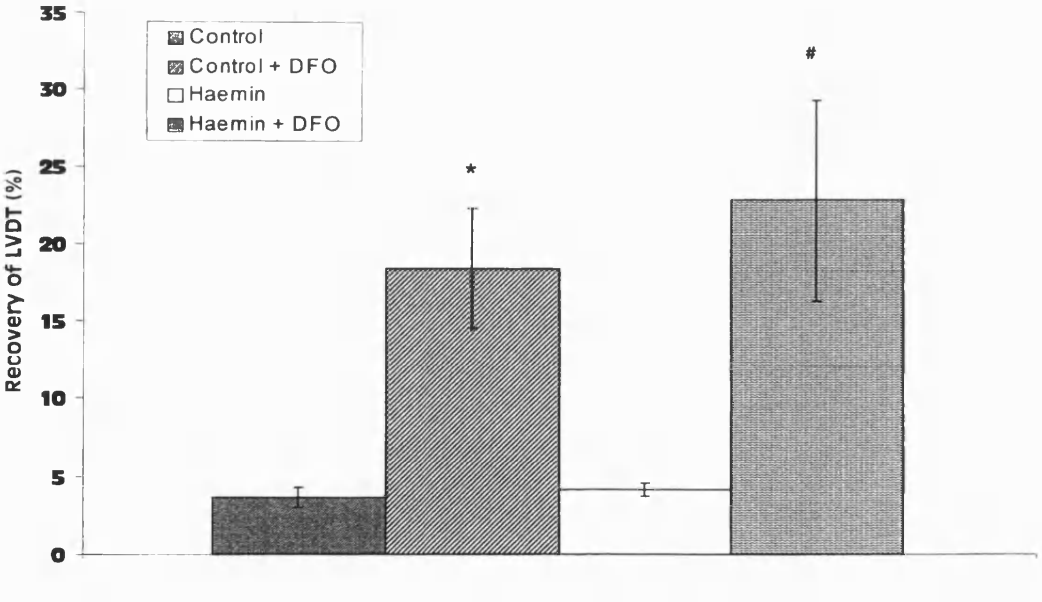
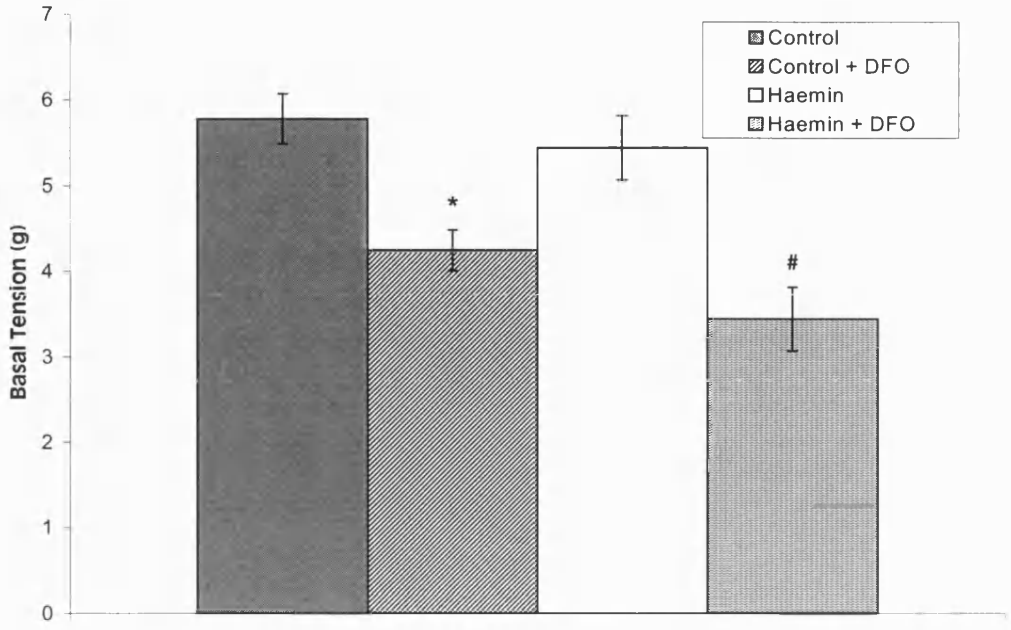


Figure 4.4.3
The effect of DFO on post-ischaemic CFR (A) and recovery of LVDT (B) in control and haemin-treated hearts perfused at a constant-pressure of 70mmHg.
Rats were treated with saline or 75 µmol/kg haemin (24hour). Values are expressed as mean ± SEM. * where $p<0.05$ compared with control and # where $p<0.05$ compared with haemin (n=5).

(A) Basal tension during reperfusion



(B) Tissue bilirubin production

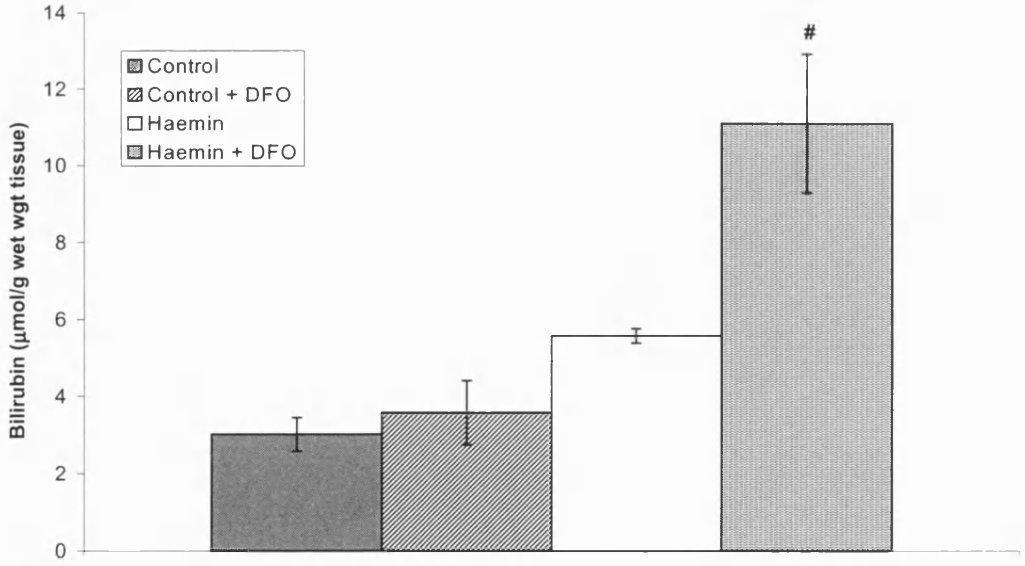


Figure 4.4.4

The effect of DFO on post-ischaemic basal tension (A) and tissue bilirubin levels (B) in control and haemin-treated hearts perfused at a constant-pressure of 70mmHg.

Rats were treated with saline or 75 µmol/kg haemin (24hour). Values are expressed as mean ± SEM. * where $p<0.05$ compared with control and # where $p<0.05$ compared with haemin (n=5).

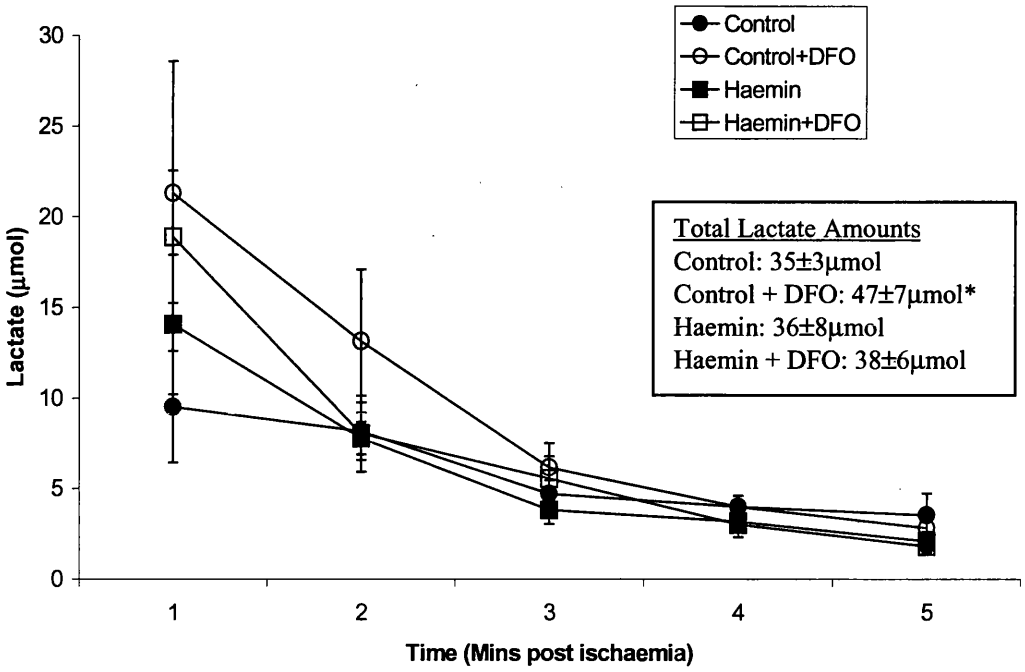


Figure 4.4.5
The effect of DFO on lactate washout after 30 min global ischaemia in control and haemin-treated hearts perfused at a constant-pressure of 70mmHg.
Rats were treated with saline or 75 μmol/kg haemin (24hour). Perfusate was collected for the initial 5 min and assayed for lactate content. Values are expressed as mean ± SEM and * $p<0.05$ compared with control (n=5). Note: some error bars may fall within the size of the symbol.

DFO does not significantly affect the washout of lactate during each minute post ischaemia in either control or haemin-treated hearts, but there is a significant increase in total lactate content of control hearts treated with DFO (Figure 4.4.5). Therefore, in control hearts, the protection conferred from DFO may be produced by an increase in anaerobic glycolysis and the accompanying rise in ATP production.

4.5. The effect of haemin on the autoregulation mechanisms of the constant-pressure-perfused rat heart.

The following experiments concentrated on investigating the effect of haemin pre-treatment on the autoregulation mechanisms involved in the control of coronary flow. It was postulated that the significant vasoconstrictor effect induced in the pre- and post-ischaemic rat hearts reported in section 4.1 could have occurred as a result of an inhibitory effect on the autoregulation mechanisms.

The data illustrated in figure 4.6.1A are a representation of the absolute CFR at the end of a 10 min period of perfusion at each pressure level. They indicate that there is no real evidence of autoregulation mechanisms in control, haemin or SnPP –treated hearts. These data are in agreement with the CFR recorded in control and haemin-treated hearts perfused at 70 mmHg and 130 mmHg. For example, the CFR is doubled in both treatment groups at 130 mmHg compared with 70 mmHg, whereas the CFR would be expected to be the same at both pressures if autoregulation mechanisms were active. Haemin and SnPP pre-treatment alone significantly reduces CFR compared with control at the lower pressure values of 100, 80 and 60 mmHg. The reduced coronary flow produced by haemin under these conditions may be dependent upon the basal pressure level. It is important to note that the large error bars on the group data for haemin + SnPP are due to the small group size of 2. The small group size occurred due to an abnormal effect on the stability of the tissue during the first pressure change (data not shown). The hearts exhibited VF after the second pressure-change to 120 mmHg in 3 of the tissues investigated. Therefore, it is difficult to ascertain the degree of involvement of HO-1 in the results described above. It could be postulated that the induction of VF occurred due to the combination of non-specific effects from haemin and SnPP. In contrast, SnPP-treated hearts demonstrated CFRs similar to those recorded in haemin-treated hearts.

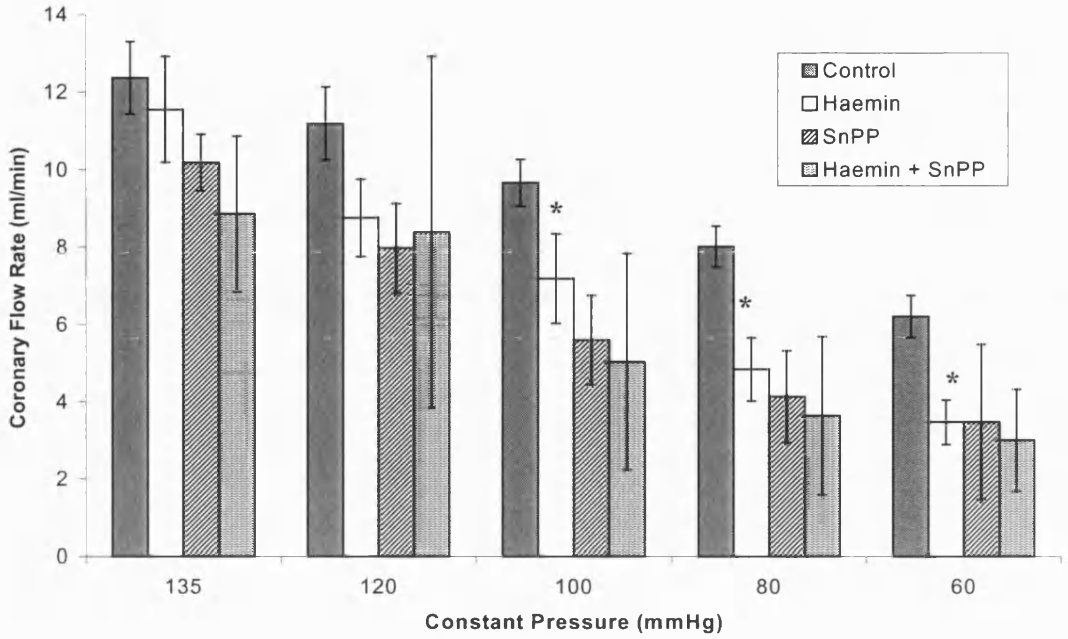
Over the initial change to 135 mmHg, there was an increase in CFR in all treatment groups except in hearts treated with haemin + SnPP (Figure 4.5.1B). The majority of recordings from the groups of hearts treated with haemin and

SnPP were taken at the pressure of 135 mmHg (n=5) prior to the VF response described earlier. From these measurements, haemin + SnPP appears to exert a combined effect by decreasing CFR compared with control. The lower CFR produced at the high pressure of 135 mmHg may indicate a reason for the resultant VF upon lowering the pressure to 120 mmHg. The most likely cause for the low CFR is an additive effect from haemin and SnPP, as both appear to lower the CFR when administered on their own.

The pressure level was further reduced from 135 mmHg to 120 mmHg and measurements taken in control, haemin- and SnPP-treated hearts over the initial 2 min period. The results illustrate that both haemin and SnPP treated hearts significantly decrease CFR compared with control over the first 20 min ($p<0.05$). This confirms the data recorded earlier, and suggests that haemin has a vasoconstrictor effect in the constant pressure rat heart. In addition, SnPP alone also produces a vasoconstrictor effect, resulting in a CFR similar to that seen in the haemin-treated heart. The inhibition and induction of HO-1 activity appears to have the same effect. This may be due to the dual mechanism of action of the metalloporphyrins, whereby HO-1 expression can be both inhibited and potentiated. Furthermore, the possibility that the VF experienced in hearts treated with haemin + SnPP is caused by an additive vasoconstrictor effect is strengthened by these data.

The further reduction in pressure from 120 mmHg to 100 mmHg (Figure 4.6.2B), 100 mmHg to 80 mmHg (Figure 4.6.3A), and 80 mmHg to 60 mmHg (Figure 4.6.3B) induced significant decreases in CFR in haemin- and SnPP-treated hearts compared with control ($p<0.05$). This result again confirms the vasoconstrictor effect of haemin and SnPP under these experimental conditions. The data indicate that the difference between haemin- and SnPP-treatment compared with control appears to increase as the pressure decreases. In addition, there do not appear to be any autoregulation mechanisms involved in the responses of control and haemin-treated hearts, as there is a gradual decrease in response over the initial 2 min of each pressure change.

(A) Absolute CFRs upon constant pressure change



(B) Initial pressure change to 135mmHg

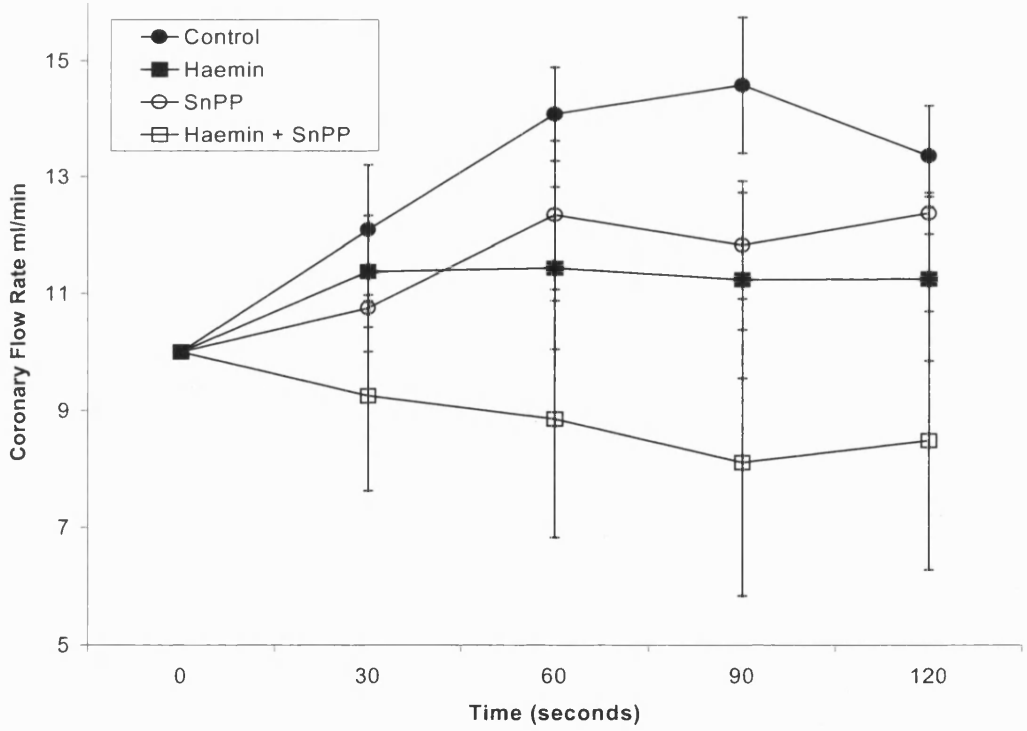
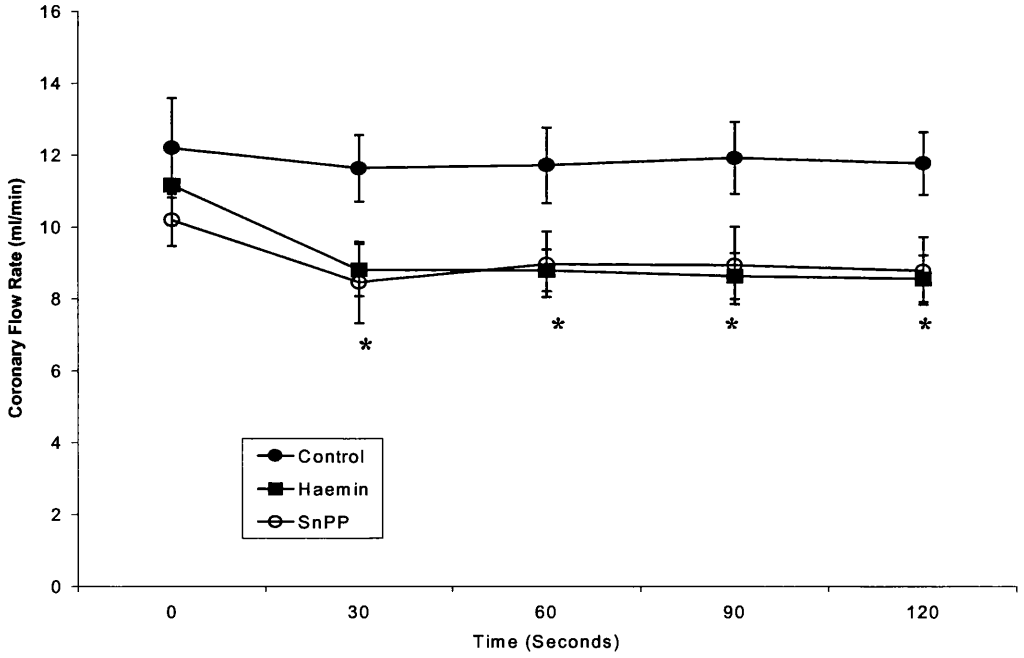


Figure 4.6.1

The effect of haemin and SnPP pre-treatment alone and in combination on absolute CFRs at various pressures (A) and CFR during the initial change to 135 mmHg (B). Rats were pre-treated with saline, 75 µmol/kg haemin (24hour) ± 40 µmol/kg SnPP (1hour) and 40 µmol/kg SnPP alone. Values are expressed as mean ± SEM. * where $p<0.05$ compared with control, n=5 (apart from haemin + SnPP n=2). Note: some error bars may fall within the size of the symbol.

(A) Pressure change from 135mmHg to 120mmHg



(B) Pressure change from 120mmHg to 100mmHg

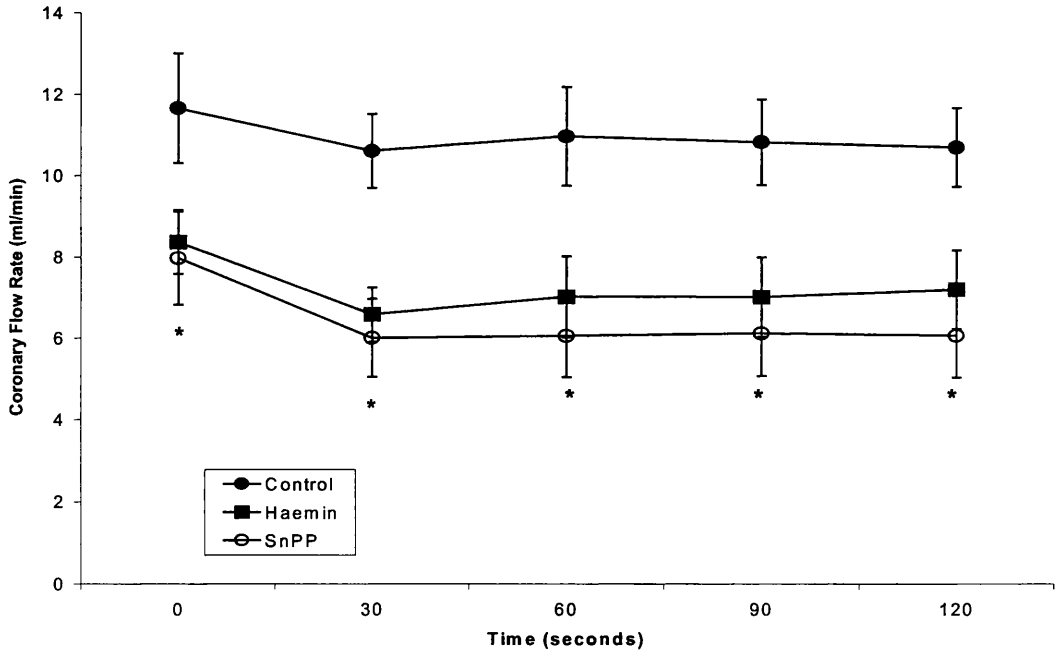
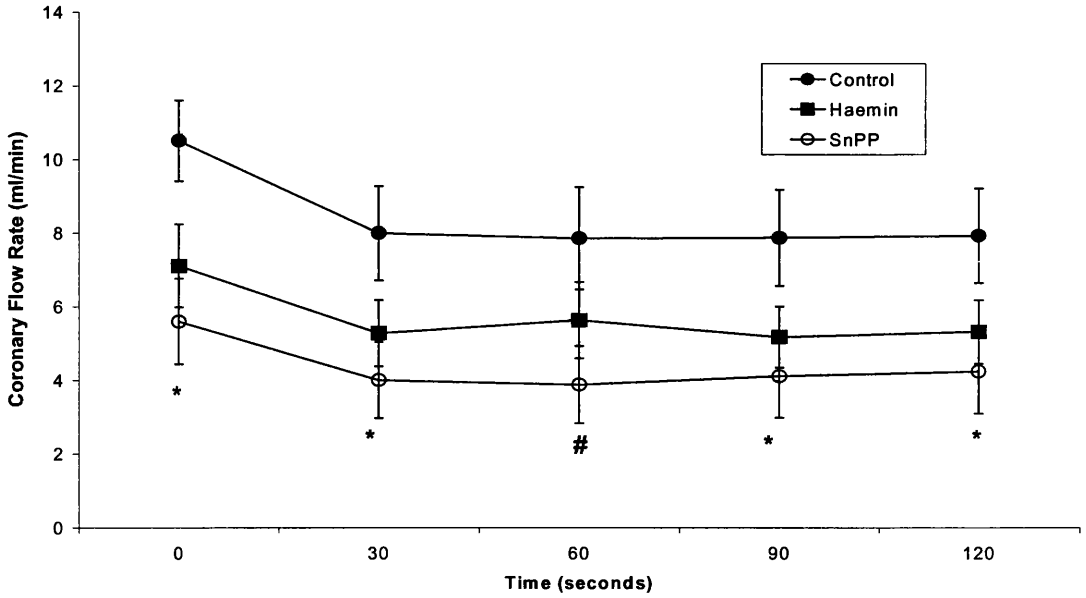


Figure 4.6.2

The effect of haemin and SnPP pre-treatment on CFR in the first 2 min after the pressure conversion from 135 mmHg to 120 mmHg (A) and 120 mmHg to 100 mmHg (B).
Rats were pre-treated with saline, 75 μmol/kg haemin (24hour) and 40 μmol/kg SnPP alone. Values are expressed as mean ± SEM. * where $p<0.05$ compared with control, (n=5).

(A) Pressure change from 100mmHg to 80mmHg



(B) Pressure change from 80mmHg to 60mmHg

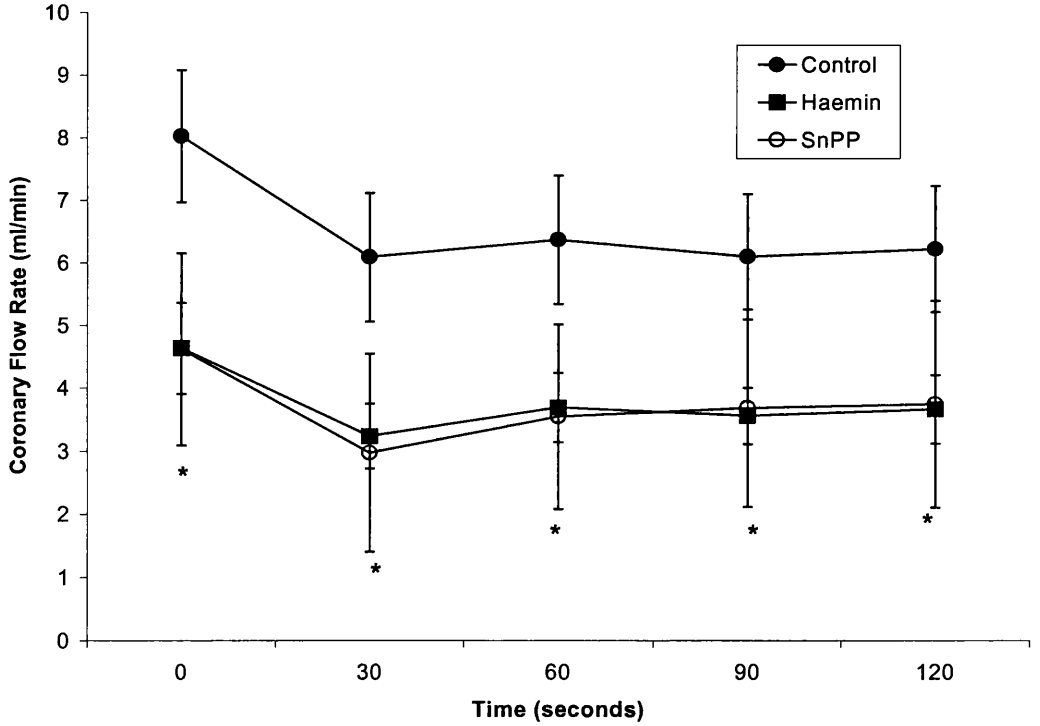


Figure 4.5.3

The effect of haemin and SnPP pre-treatment on CFR in the first 2 min after the pressure conversion from 100 mmHg to 80 mmHg (A) and 80 mmHg to 60 mmHg (B).

Rats were pre-treated with saline, 75 μmol/kg haemin (24hour) and 40 μmol/kg SnPP alone. Values are expressed as mean ± SEM. * $p<0.05$ in haemin-treated hearts compared with control, # $p<0.05$ SnPP alone compared with control (n=5).

4.6 Chapter 4: Summary

- At 70 mmHg, haemin pre-treatment produced a reduction in CFR, indicative of a vasoconstrictor response. SnPP significantly reduced the force of contraction and haemin treatment produced a significant reduction in heart rate.
- Haemin had no effect on CFR during reperfusion, although haemin + SnPP significantly reduced CFR. Recovery from I/R occurred in both control and haemin-treated hearts to a similar extent. SnPP abolished the recovery of contractility in control and haemin-treated hearts.
- Increasing ischaemic insult abolished reactive hyperaemia and recovery of contractility, although the vasoconstrictor effect was present during reperfusion of haemin-treated hearts. Tissue bilirubin levels increased in haemin-treated hearts compared with control after 30 min I/R.
- At 130 mmHg, haemin produced a vasoconstrictor effect and a reduction in contractility and heart rate compared with hearts perfused at 70 mmHg.
- During reperfusion, the CFR was markedly increased in control and haemin-treated hearts compared with those perfused at 70 mmHg, but the constrictor effect of haemin was also present. The recovery of cardiac contractility was significantly increased in haemin-treated hearts compared with control at 130 mmHg.
- DFO significantly increased CFR and decreased the force of contraction in both control and haemin-treated hearts.
- During reperfusion DFO significantly increased the CFR in control hearts. Recovery of contractility was increased in both control and haemin-treated hearts perfused with DFO.
- The vasoconstrictor effect of haemin did not appear to be produced by the modulation of autoregulatory mechanisms.

CHAPTER 5:
THE EFFECT OF HAEMIN PRETREATMENT ON VASCULAR RESPONSIVENESS IN THE PERFUSED KIDNEY AND MESENTERY

Chapter 5: The effect of haemin pre-treatment on vascular responsiveness in the perfused mesentery and kidney.

5.1 The effect of haemin pre-treatment on vascular responsiveness in the perfused mesentery

Figure 5.1.1 shows a representative trace illustrating the effect of bolus doses of histamine in the pre-contracted kidney (Trace A), the effect of bolus doses of ACh in the pre-contracted mesenteric vascular bed (Trace B) and the effect of bolus doses of ET-1 on the perfused mesenteric vascular bed (Trace C).

5.1.1. The effect of haemin pre-treatment on the response to vasodilation in the perfused mesentery.

The mesentery preparation was cannulated as described in chapter 2, and perfused for 30 min prior to addition of 10 μM PE, to increase basal tone. The initial perfusion pressure was measured after 30 min perfusion in both control and haemin-treated mesentery preparations (Figure 5.1.2A).

Haemin pre-treatment did not significantly increase basal perfusion pressure ($p>0.05$, Figure 5.1.2A). Once tissue viability had been checked, the vascular tone of the tissue was increased using the α1-agonist phenylephrine in order to investigate the effect of haemin pre-treatment on vasodilation. Haemin pre-treatment significantly reduced the vasoconstrictor effect of PE compared with control ($p<0.05$, Figure 5.1.2B). If HO-1 expression were increased in haemin-treated tissues, it seems probable that this reduction would be due either to increased CO production after prolonged contraction, or to a non-specific side-effect of haemin treatment as yet unidentified.

Responses to bolus injections of 0.1-30 nmol ACh were abolished in haemin-treated but not control samples ($p<0.05$) (Figure 5.1.3A), while injections of histamine elicited dose-dependent vasodilatory responses in both control and haemin-treated tissues (Figure 5.1.3B). However, responses were smaller in absolute terms in the latter. Thus, the effect of haemin treatment on histamine-induced vasodilation was less marked than its effect on ACh-induced dilatation.

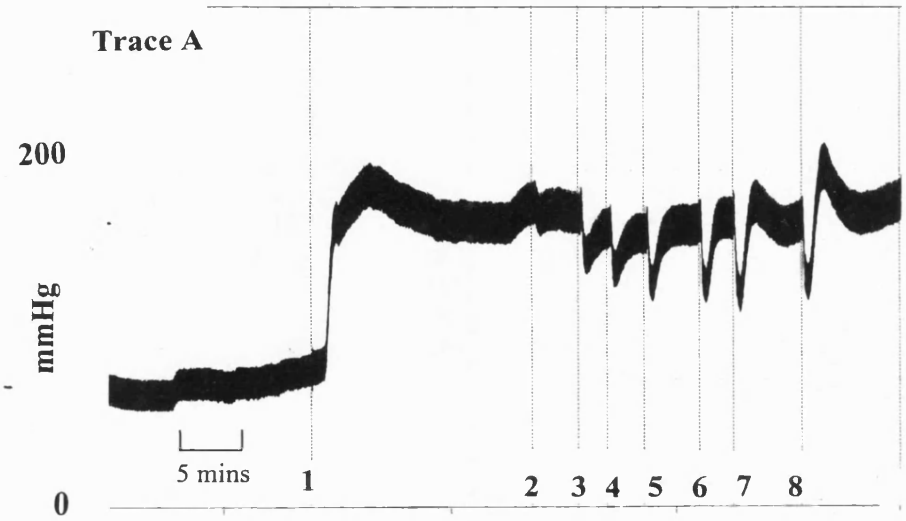


Figure 5.1.1A

This is a representative trace of the isolated perfused rat kidney pre-contracted with 30mM K^+ (1), followed by dose responses to histamine (0.1 to 100nmol, 2-8).

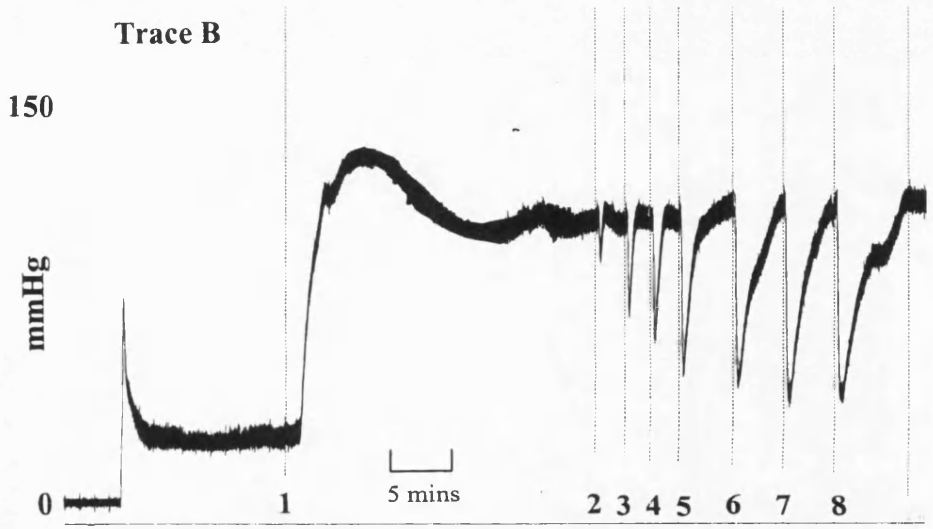


Figure 5.1.1B

This is a representative trace of the isolated perfused rat mesenteric vascular bed pre-contracted with 10μM Phenylephrine (1), followed by dose responses to ACh (0.1 to 100nmol, 2-8).

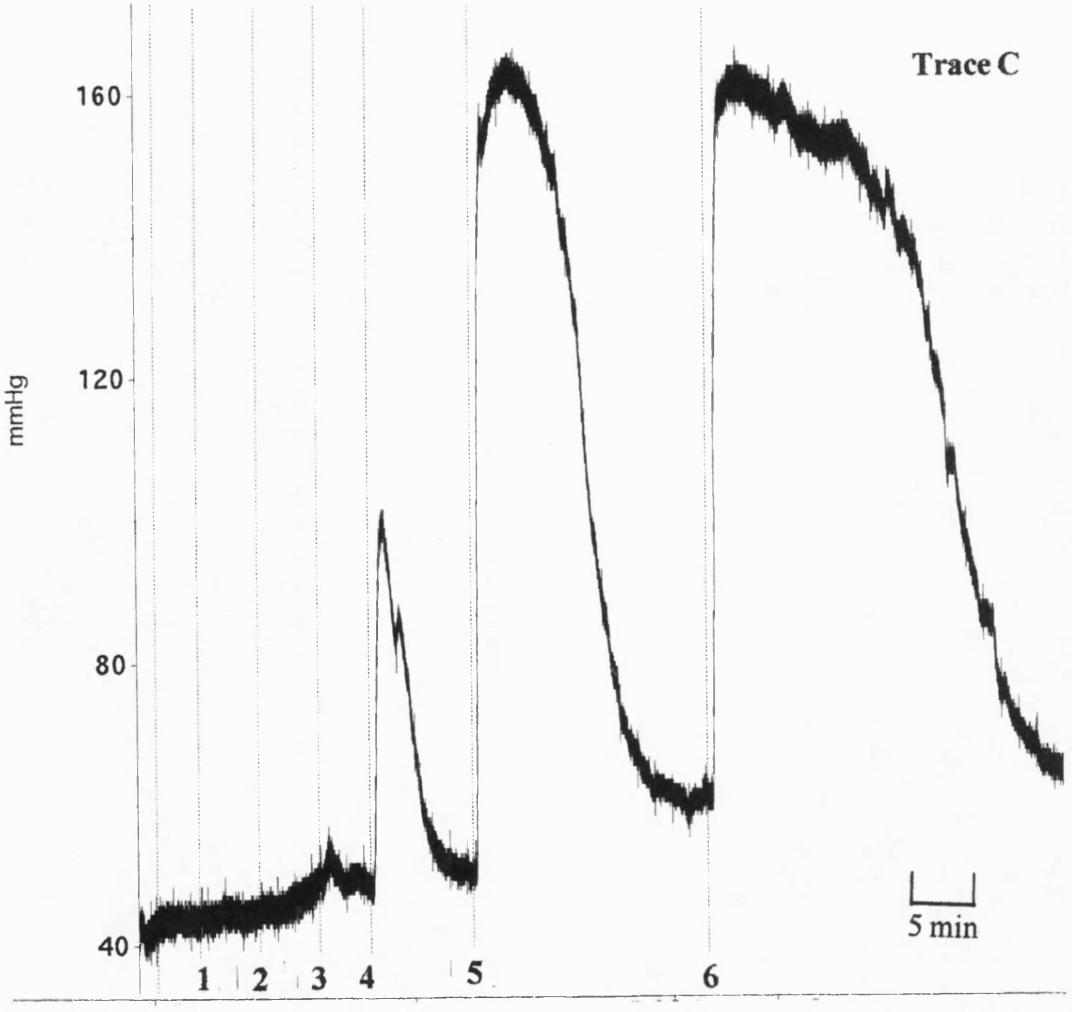
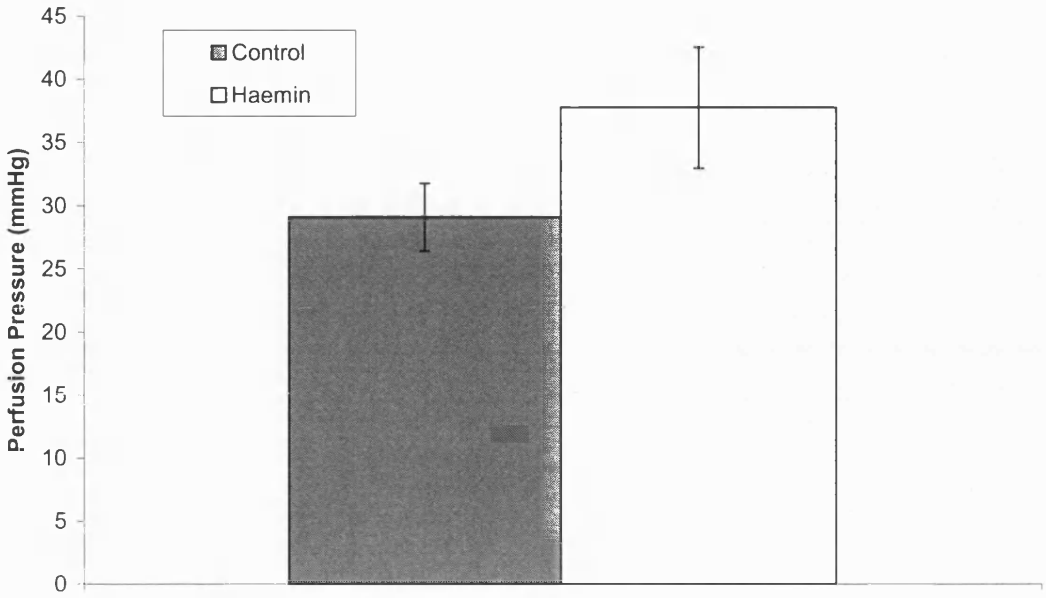


Figure 5.1.1 C

This is a representative trace of the response to bolus doses of ET-1 over a range of 1 to 300pmol (no. 1-6) in the perfused rat mesenteric vascular bed.

(A)



(B)

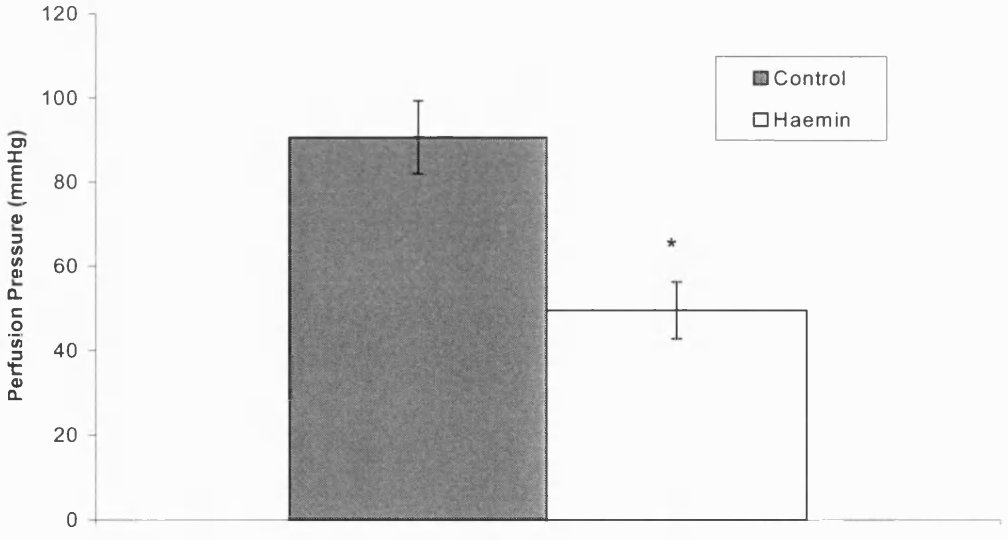
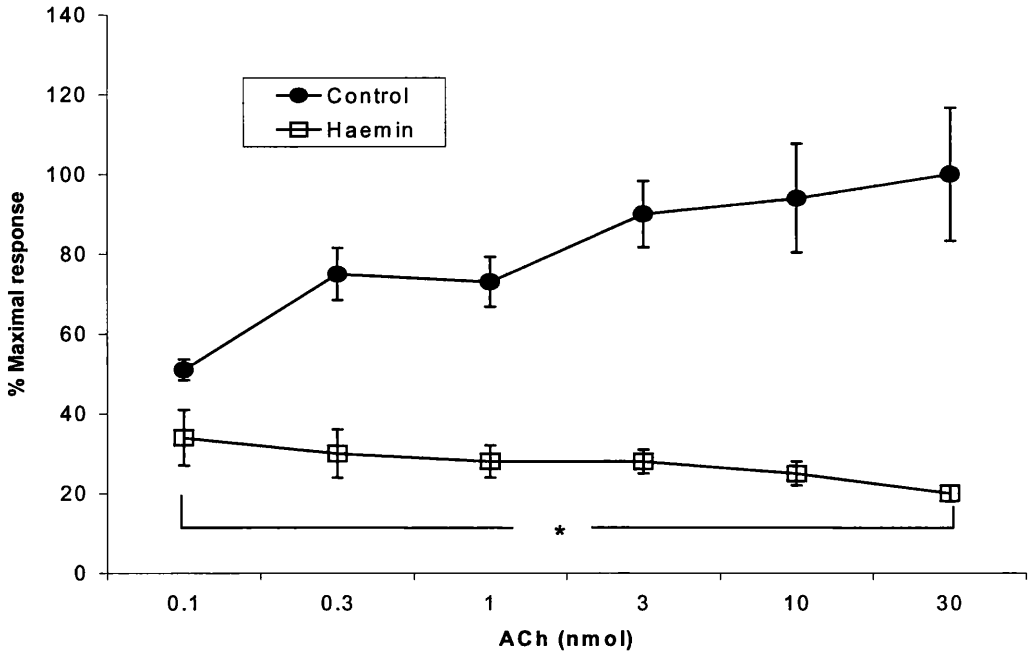


Figure 5.1.2
The effect of haemin pre-treatment on the basal perfusion pressure (A) and the increased vascular tone induced by Phe infusion (B) in the perfused mesenteric vascular bed.
Male wistar rats were pre-treated with saline or 75 µmol/kg haemin. Values are expressed as mean ± SEM and * $p<0.05$ compared with control (n=5-15).

It is important to note that it was very difficult to maintain the pre-contraction perfusion pressure in haemin-treated tissues, as there was a gradual reduction in perfusion pressure after exposure to 2-3 dose response cycles with different vasodilator agents.

(A)



(B)

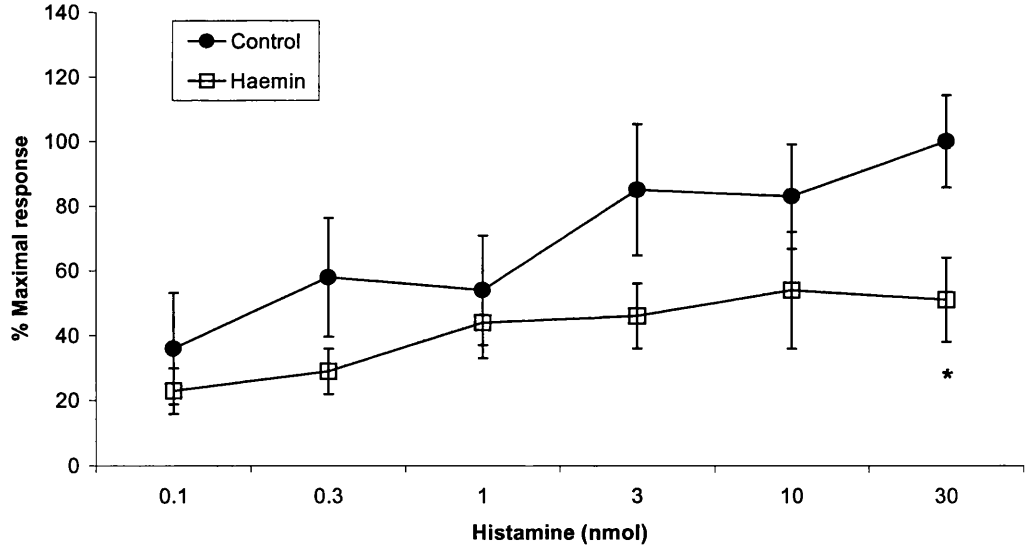


Figure 5.1.3

The effect of haemin pre-treatment on the vascular responsiveness to bolus injections of ACh (A) and histamine (B) in the pre-contracted mesentery.

Male wistar rats were pre-treated with saline or 75 µmol/kg haemin (24hour). Bolus injections of ACh, and histamine were investigated over a cycle period of 5 min. Values are expressed as mean ± SEM. * where $p<0.05$ compared with control (n=5). Note: some error bars may fall within the size of the symbol.

5.1.2. The effect of haemin pre-treatment on the response to vasoconstrictor agents in the perfused mesentery.

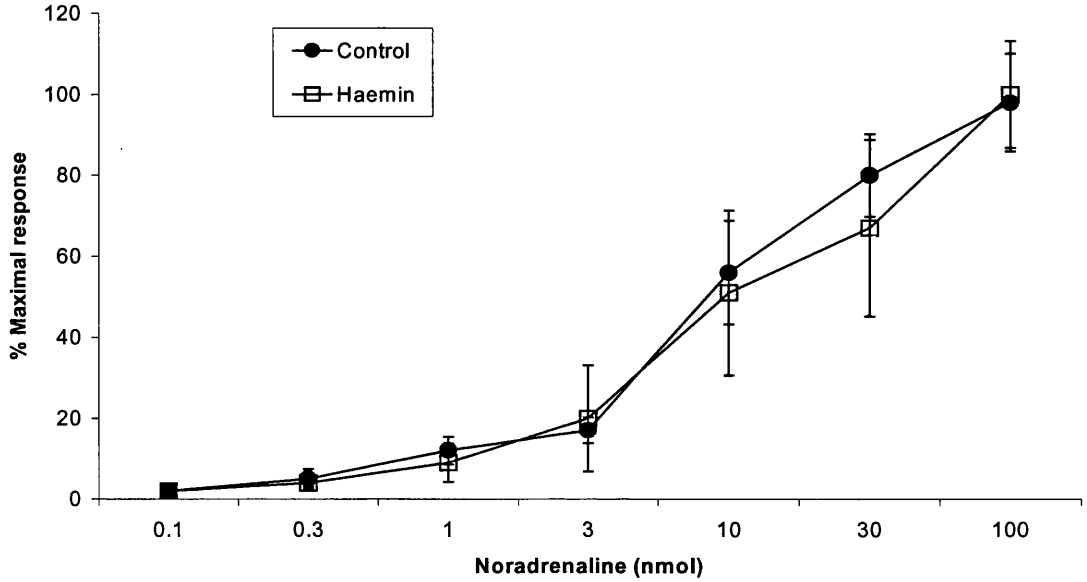
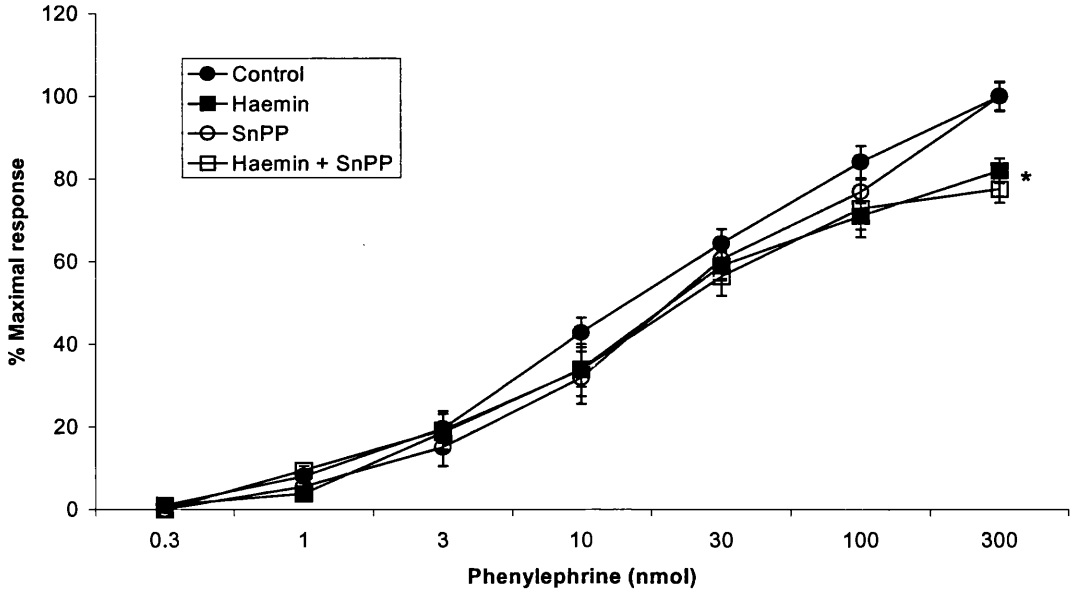


Figure 5.1.4
The effect of haemin pre-treatment on the vascular responsiveness to bolus injections of noradrenaline.
Male wistar rats were pre-treated with saline or 75 μmol/kg haemin (24hour). Bolus injections of NA were investigated over a cycle period of 5 min. Values are expressed as mean ± SEM (n=5). Note: some error bars may fall within the size of the symbol.

To investigate the reactivity of the vessels cannulated, vascular responsiveness was monitored by measuring responses to NA (Figure 5.1.4). A dose-response curve can be drawn to illustrate how vascular responsiveness increased in response to bolus injections of 0.1-100 nmol NA in both control and haemin-treated tissues. Haemin pre-treatment did not significantly affect the response to NA, indicating that haemin does not block α_1 adrenoceptors.

(A)



(B)

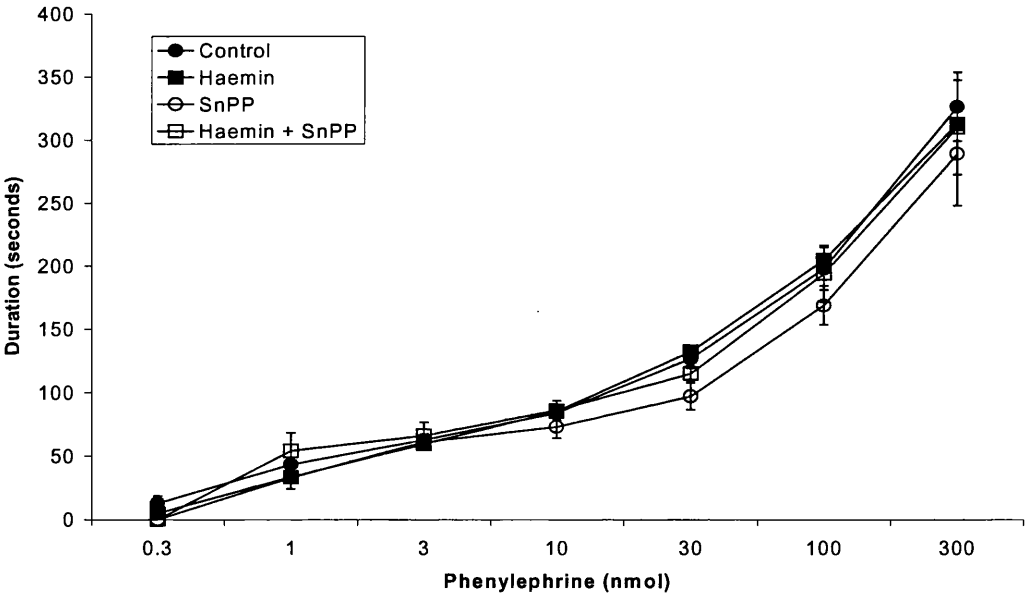
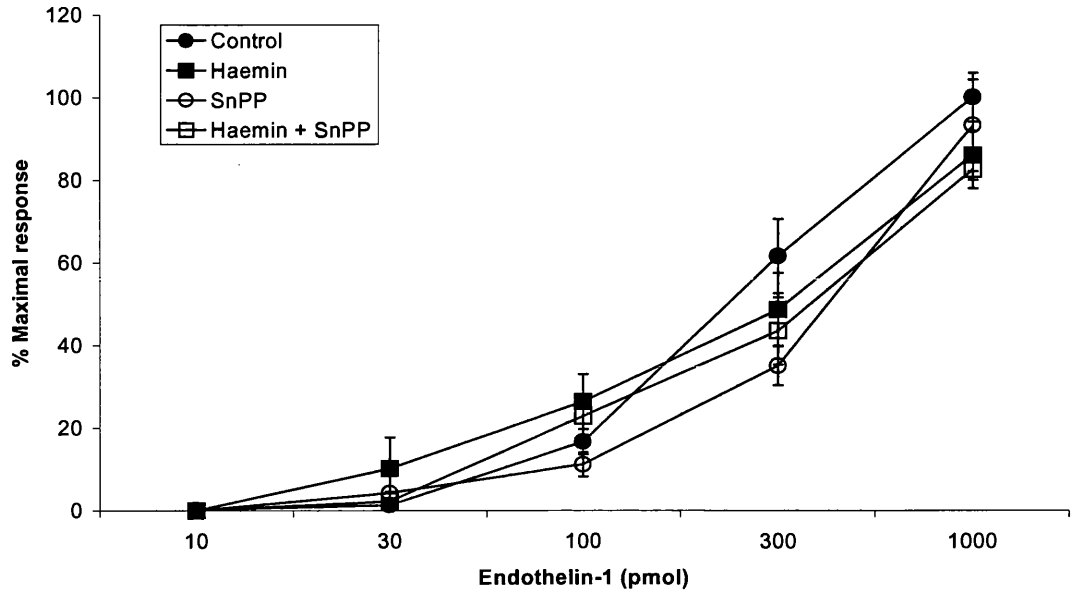


Figure 5.1.5

The effect of haemin and SnPP pre-treatment alone and in combination on the vascular responsiveness to bolus doses of phenylephrine in the perfused mesentery.

Male wistar rats were pre-treated with saline, 75 μmol/kg haemin (24hour) and 40 μmol/kg SnPP or haemin + SnPP. The peak height of the increase in perfusion pressure (A) and duration (B) were calculated, and values are expressed as mean ± SEM. * where $p<0.05$ compared with control and SnPP alone (n=5). Note: some error bars may fall within the size of the symbol.

(A)



(B)

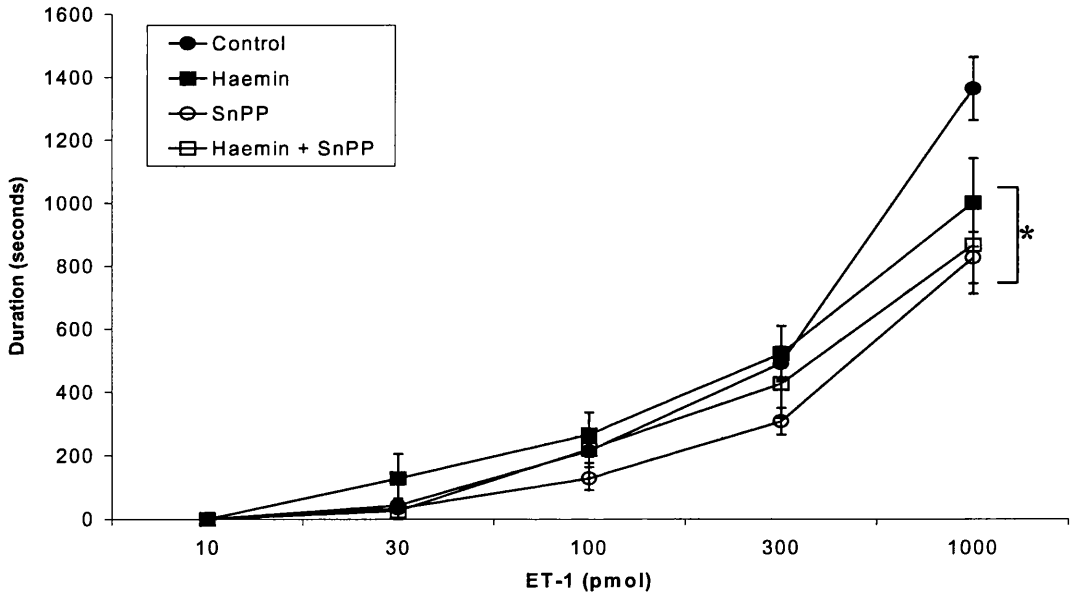


Figure 5.1.6

The effect of haemin and SnPP pre-treatment alone and in combination on the vascular responsiveness to bolus doses of ET-1 in the perfused mesentery.

Male wistar rats were pre-treated with saline, 75 μmol/kg haemin (24hour) and 40 μmol/kg SnPP or haemin + SnPP. The peak height of the increase in PP (A) and duration (B) were calculated, and values are expressed as mean ± SEM. * where $p<0.05$ compared with control (n=5). Note: some error bars may fall within the size of the symbol.

Haemin pre-treatment (± SnPP) did not significantly affect the vasoconstrictor response induced by PE, as compared with responsiveness in control tissue and tissue treated with SnPP alone (Figure 5.1.5A). Furthermore, neither haemin nor SnPP affected the duration of the PE-induced vasoconstrictor response (Figure 5.1.5B).

The effect of haemin pre-treatment on ET-1-induced vasoconstriction was also investigated. Results from these investigations indicated that neither haemin nor SnPP exerted a significant effect on the peak response to any of the doses of ET-1 used (Figure 5.1.6A). However, the duration of action of ET-1 (1nmol) was significantly reduced in tissues treated with haemin alone, SnPP alone and haemin + SnPP (Figure 5.1.6B). This could be another demonstration of the non-specific effects of haemin and SnPP. Angiotensin II was found to have no effect on perfusion pressure in either control or haemin-treated hearts (data not shown).

5.2 The effect of haemin pre-treatment on vascular responsiveness in the perfused kidney

5.2.1. The effect of haemin pre-treatment on the vasodilator activity of the perfused kidney.

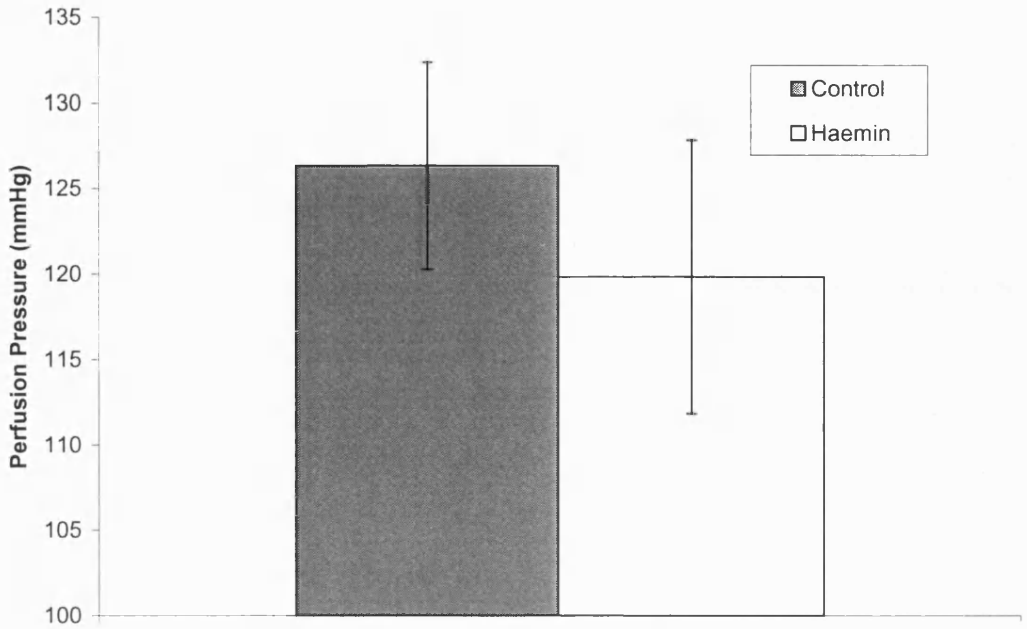
The effects of vasodilators were also determined in the isolated perfused rat kidney. Kidneys were removed from rats treated with saline, 75 μmol/kg haemin (24hours) ± 40 μmol/kg SnPP (1hour) or SnPP alone. The tissue was perfused for 30 min before vascular tone was increased so that the effects of vasodilator substances could be investigated. The vascular tone was increased by perfusing with Krebs solution containing 30 mM K^+.

Haemin treatment did not significantly affect the basal perfusion pressure of the perfused kidney ($p>0.05$, Figure 5.2.1A). After 30 min perfusion with normal Krebs, the perfusate was changed to Krebs containing 30 mM K^+ in order to induce an increase in perfusion pressure. This solution was used in the case of the kidney because it was very difficult to maintain a stable perfusion pressure with PE in the kidney. After pre-contracting the tissue with 30 mM K^+, there was no significant difference in the perfusion pressure of either control or haemin-treated hearts after 30 min (Figure 5.2.1B).

The first agent tested was histamine, which was used because it produced the best dose-response curve from all the vasodilators investigated (Figure 5.2.2A). In kidneys pre-treated with haemin, the dose-dependent response to histamine was not significantly different from that seen in controls ($p>0.05$).

In contrast, it was very difficult to achieve a gradual dose response curve to ACh in either control or haemin-treated kidneys (Figure 5.2.2B). Thus, we can conclude that haemin pre-treatment did not produce any significant effect compared with controls.

(A)



(B)

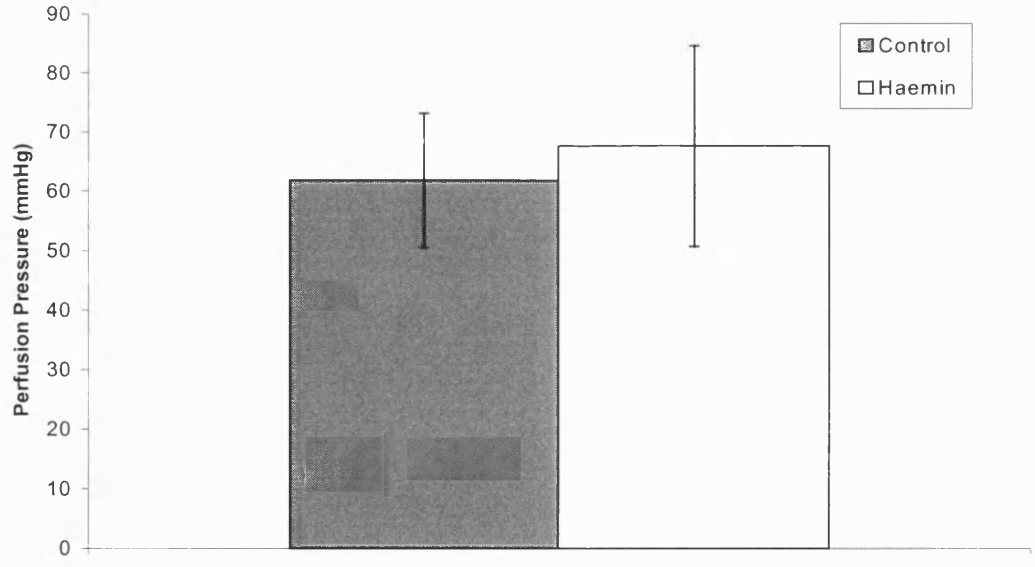
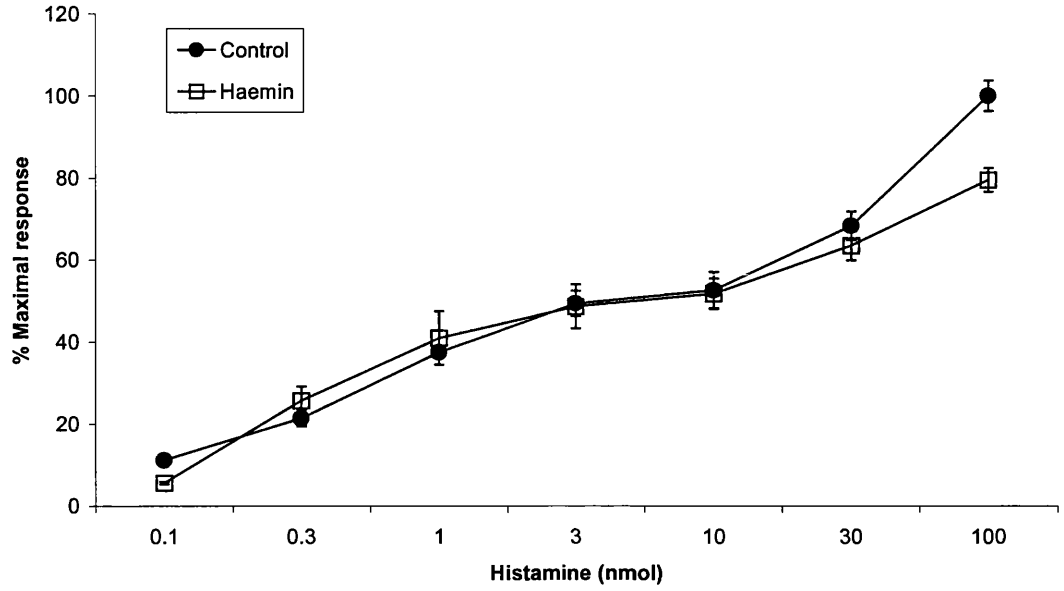


Figure 5.2.1
The effect on haemin pre-treatment on the basal perfusion pressure (A) and perfusion pressure of perfused kidney preparations pre-contracted with Krebs containing 30 mM K^+(B). Values are expressed as mean ± SEM (n=15).

(A)



(B)

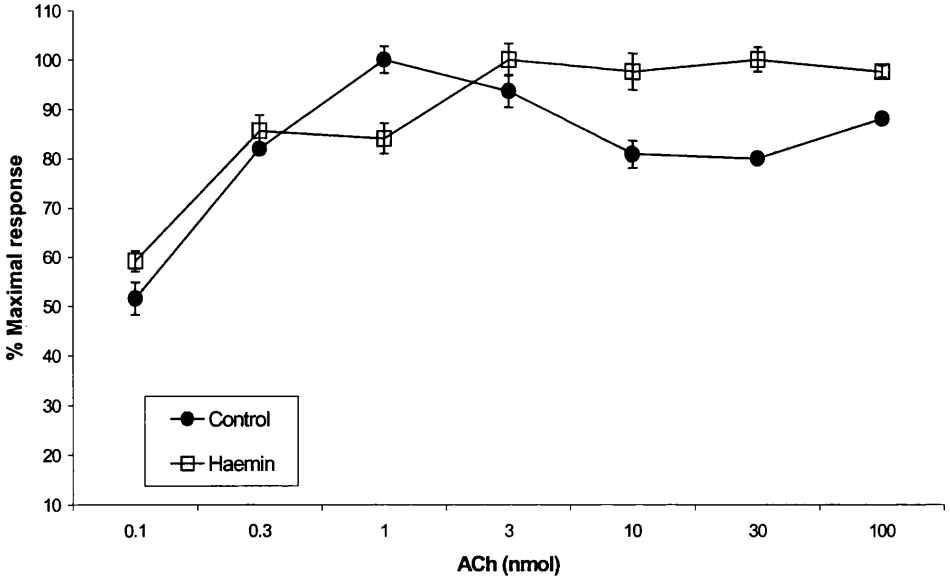


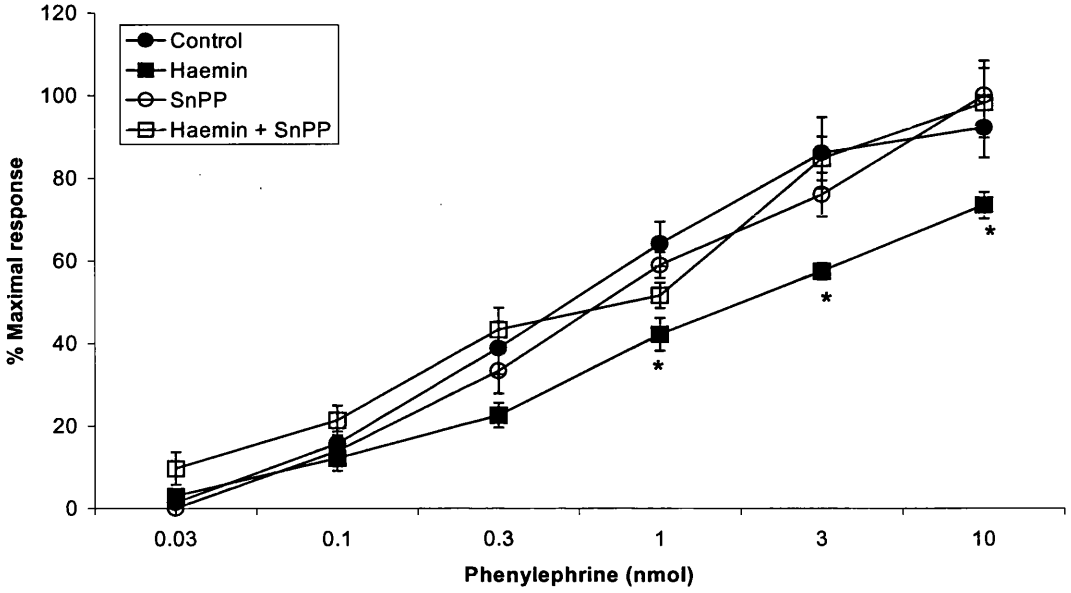
Figure 5.2.2

The effect of haemin pre-treatment on the vascular responsiveness to bolus injections of histamine (A) and ACh (B) in the K+ pre-contracted kidney.

Bolus injections of ACh and histamine were investigated over a cycle period of 5 min. Values are expressed as mean ± SEM (n=10). Note: some error bars may fall within the size of the symbol.

5.2.2. The effect of haemin pre-treatment on the response to vasoconstrictor agents in the perfused kidney.

(A)



(B)

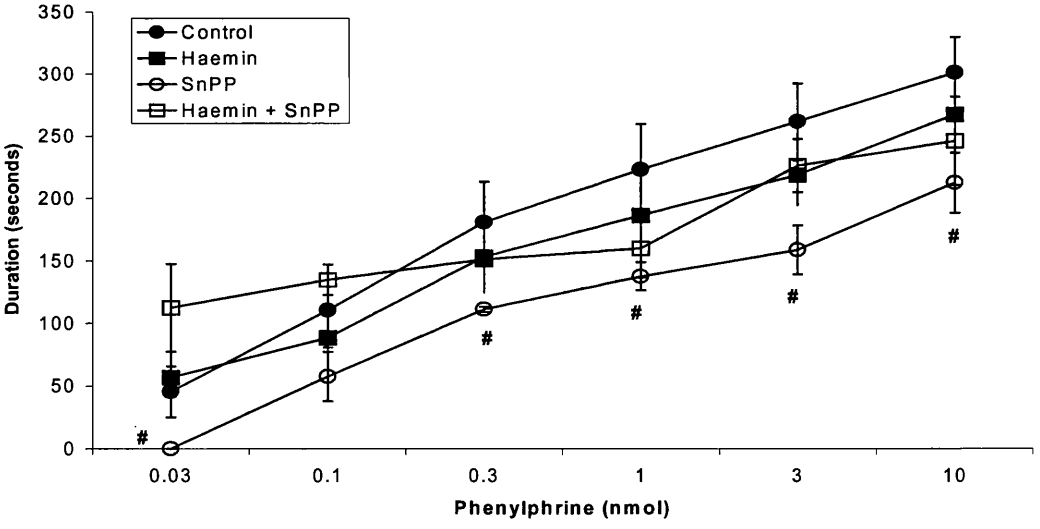


Figure 5.2.4

The effect of haemin and SnPP pre-treatment on the vascular responsiveness to phenylephrine in the perfused kidney.

Rat kidneys were pre-treated with saline, 75μmol/kg haemin ± 40μmol/kg SnPP or 40μmol/kg SnPP alone.

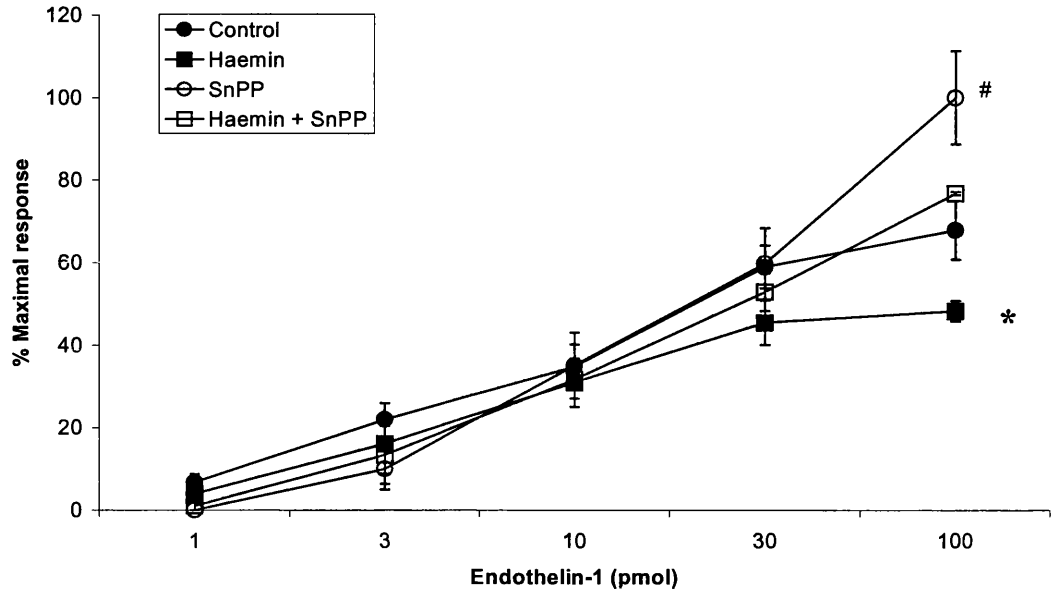
Bolus injections of PE were investigated over a cycle period of 5 min. The peak height (A) and duration (B) were calculated, and values are expressed as mean ± SEM. * where $p<0.05$ compared with control and # compared with haemin (n=5). Note: some errors may fall with the symbol size.

Figure 5.2.4 shows the effect of haemin ± SnPP on renal vasoconstrictor responses to bolus doses of PE. Haemin pre-treatment significantly reduced responses to PE at higher doses such as 1, 3 and 10 nmol. In contrast, SnPP treatment significantly reduced the duration of response to PE compared with control (Figure 5.2.4B). Therefore, these data suggest that inhibition of HO decreased the length of response to PE.

The effect of haemin pre-treatment on the dose response to ET-1 was also investigated. Haemin pre-treatment significantly reduced the response to ET-1 at the highest dose tested, whereas SnPP significantly increased the response to 100 pmol ET-1 (Figure 5.2.5A). Haemin pre-treatment significantly reduced the duration of response at the top end of the dose response curve, while SnPP significantly reduced duration of response at the lower end of the dose-response curve (Figure 5.2.5B).

Finally, the effect of haemin pre-treatment was examined on the response to sarafotoxin-6c (SX6C), an ET_B agonist (Figure 5.2.6A and B). The only significant effect on vascular responsiveness induced by haemin on SX6C, was observed at the lowest dose of 1pmol and there was no significant difference in the duration of response to SX6C in control or haemin-treated kidneys.

(A)



(B)

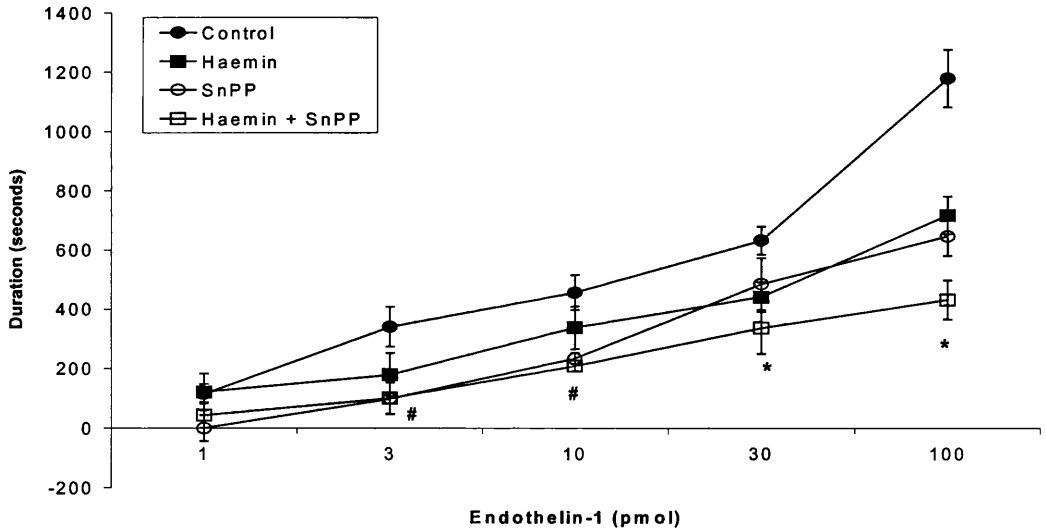


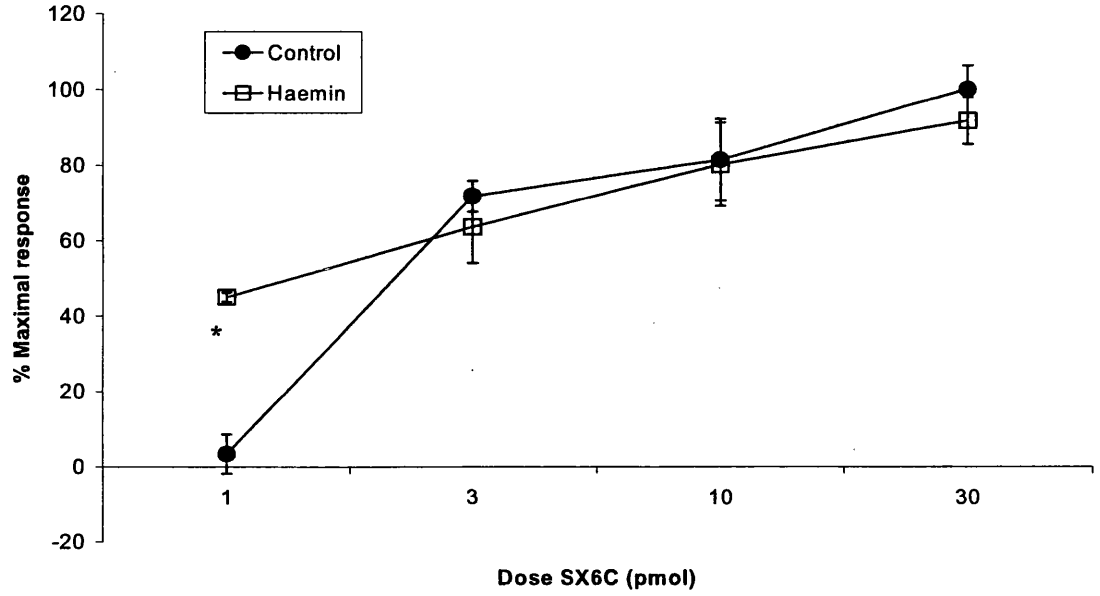
Figure 5.2.5

The effect of haemin and SnPP pre-treatment on the vascular responsiveness to endothelin-1 (ET-1) in the perfused kidney.

Rat kidneys were pre-treated with saline, 75μmol/kg haemin ± 40μmol/kg SnPP or 40μmol/kg SnPP alone.

Bolus injections of ET-1 were investigated over a cycle period of 5 min. The peak height (A) and duration (B) were calculated, and values are expressed as mean ± SEM. * where $p<0.05$ haemin compared with control or # haemin + SnPP / SnPP compared with control (n=5). Note: some errors may fall with the symbol size.

(A)



(B)

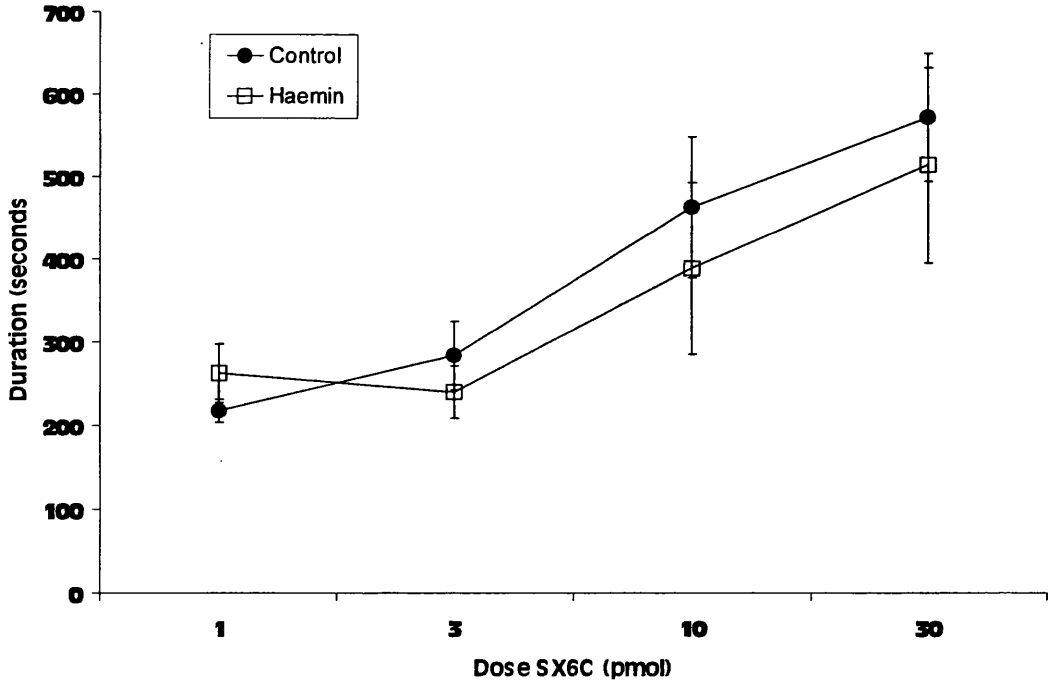


Figure 5.2.6

The effect of haemin and SnPP pre-treatment on the vascular responsiveness to sarafotoxin-6c (SX6C) in the perfused kidney.

Rat kidneys were pre-treated with saline or 75μmol/kg haemin (24 hour).

Bolus injections of SX6C were investigated over a cycle period of 5 min. The peak height (A) and duration (B) were calculated, and values are expressed as mean ± SEM (n=5). Note: some errors may fall with the symbol size.

CHAPTER 6:
DISCUSSION

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6.1. Determination of methods to evaluate HO-1 expression and release of HO products

The investigation of the effect of increased HO-1 expression in the constant flow rat heart was carried out by pre-treating rats with haemin. Haemin has been demonstrated to increase expression of HO-1, as it is the substrate for HO, and the resulting increase in availability of haem results in induction of HO-1 expression (Maines 1997, Clark et al., 2000). It was established that treatment with 75 μmol/kg haemin (24 hour) sufficiently increased HO-1 protein expression in the rat heart. SnPP did not inhibit the induction of HO-1 expression by haemin, but may act to inhibit HO activity.

The measurement of a product from haem catalysis was important for the identification of possible mediators involved in the haemin-induced responses and as an estimation of HO activity. Therefore, the method by Van Roy et al., (1971) was employed and modified for the measurement of tissue bilirubin content. As the tissue was freeze-clamped at the end of each experiment, tissue bilirubin levels can only be commented upon with regards to I/R injury.

6.2 The effect of haemin-induced HO-1 expression on the pre-ischaemic rat heart.

6.2.1 The effect of haemin pre-treatment on the constant flow-perfused rat heart.

In the pre-ischaemic heart, haemin pre-treatment produced a vasodilator effect in a dose dependent manner compared with control. The most likely cause for this reduction in CPP is the release of CO via the HO-mediated catalysis of haemin; it should be noted that CO is a known vasodilator (Durante & Schafer, 1998, Kozma et al., 1999). Thus, increased availability of the substrate haem leads to increased breakdown and the release of CO, biliverdin and iron. In the presence

of SnPP, these data implicate that the vasodilator response induced by HO is only partially inhibited by SnPP, but it is also possible that other vasodilator mediators such as NO or PGI_2 are involved in the response. Leffler et al., (2001) have suggested that an interaction between these modulators is plausible due to the close juxtaposition of NOS, COX and HO in the vasculature. Furthermore, the dilator effect may be produced as a result of the inhibition of a vasoconstrictor mediator, such as ET-1. For example, Coceani et al., (1997) reported that CO-induced dilation in the lamb ductus arteriosus was induced by the inhibition of ET-1 production.

Haemin pre-treatment did not affect the contractility or heart rate of the tissue suggesting that HO-1 up-regulation may not have any impact on the control of contractility in the rat heart perfused under constant flow conditions. However, HO-2 and lower, basal levels of HO-1 do appear to be involved in the regulation of contractility and heart rate, as indicated in rat hearts treated with SnPP alone. The non-specific inhibition of guanyl cyclase by SnPP (Grundemar & Ny, 1997) may result in a decrease in cardiac cGMP levels. This could also be explained as a result of the direct inhibition of HO-2, which is the constitutive form of the HO. This might also be expected to decrease cardiac cGMP levels, as CO released via the HO-2-mediated catalysis of haem produces its effect by activating guanyl cyclase and increasing cGMP levels, and could therefore account for the increased force of contraction. However, in Kelly's experiments the rat heart, gave a very different result: an increase in the force of contraction produces a reduction in heart rate, a phenomenon known as the negative staircase effect (Kelly & Hoffmann, 1960).

6.2.2 The effect of haemin-induced HO-1 expression in the constant-pressure-perfused rat heart.

At a constant pressure of 70 mmHg, haemin pre-treatment significantly reduced CFR indicating a vasoconstrictor response. These data are in opposition to data recorded at constant-flow. Furthermore, the vasoconstrictor effect of haemin pre-treatment conflicts with the results of other studies where HO-1 induction has been reported to induce a vasodilator response due to the release of CO

(Furchgott & Jothianandan, 1991, McFaul & McGrath, 1987). However, a report by Imai et al., (2001), has shown that the selective overexpression of HO-1 in mouse transgenic VSMCs significantly increased blood pressure, due to an inhibitory effect on NO-induced vasodilatation. It is possible that the induction of HO-1 expression by haemin may have an inhibitory effect on the natural vasodilator control mechanisms in the constant-pressure-perfused rat heart.

SnPP did not inhibit the effect of haemin pre-treatment on the CFR. This may be for a number of reasons. Firstly, HO-1 may not be completely inhibited by the dose of SnPP selected. Secondly, the effect could be produced as a result of some non-specific action of haemin; thus, as the response was not completely inhibited by SnPP, this could suggest the involvement of other vasoactive mediators. (For example, haemin treatment has been shown to increase iNOS expression and NO release (Suzuki et al., 1995). Thirdly, the involvement of further vasoactive mediators may prevent complete inhibition. For example, in the gracillis muscle arteriole, the constrictor effect of SnPP was increased after NOS inhibition (Johnson et al., 2002). Furthermore, HO-2 is unlikely to be involved in the endogenous control of CFR in control hearts perfused at a constant pressure of 70 mmHg (as was indicated by the results of experiments using SnPP alone).

HO-1 induction does not appear to affect the force of contraction in the pre-ischaemic rat heart perfused at constant pressure. This result is surprising as the dilator response might be expected to induce a reduction in the force of contraction, according to the Gregg phenomenon (Gregg, 1963). Furthermore, SnPP significantly reduced the force of contraction in both control and haemin-treated hearts, and this was not related to an effect on heart rate. Therefore, the lack of selectivity of SnPP in the presence and absence of haemin suggests that this effect may be due to a non-specific effect from SnPP treatment. Metalloporphyrins have been shown to induce cell death as a result of ROS generation (Ohse et al., 2001), a phenomenon that would affect the force of contraction in the pre-ischaemic rat heart regardless of haemin pretreatment. However, this effect would be expected to become potentiated in the presence of haemin, which is another agent of ROS generation (Schmitt et al., 1993).

6.2.3 The effect of NO and COX products in the pre-ischaemic constant-flow perfused rat heart.

L-NO-Arg significantly increased CPP in haemin pre-treated hearts, suggesting that NO release is important in the vasodilator response to haemin pre-treatment. The dual involvement of NO and CO have been previously demonstrated in the ischaemic heart, where the release of CO and NO together serves to potentiate their effects on cGMP generation (Maulik et al., 1996). For example, an induction of HO-1 expression and an increase in CO release may combine to increase the formation of NO, thus potentiating the response to haemin alone. However, L-NO-Arg did not affect CPP in control hearts. This is an unexpected result as previous studies have demonstrated a reduced coronary flow and an increased CPP in the constant-pressure and constant-flow-perfused heart systems respectively after NOS inhibition (Kodja et al., 1997). Moreover, NOS inhibition has been shown to reduce the hyperaemic response, and to enhance the auto-regulation of blood flow (Pohl & de Wit., 1999).

L-NO-Arg had little effect on LVDT in haemin-treated hearts, indicating that NO release does not appear to be important in the control of contractility in this system. This is in agreement with a study by Nawrath et al., (1995) where NO donors were shown to have little effect on myocardial contractility in heart muscle preparations from guinea pig, rat, rabbit and man. In the normal rat heart, it has been postulated that endogenous NO has a positive inotropic effect (Kodja et al., 1997). In order to account for the fact that NOS inhibition had little effect on contractility, the following possible explanations: 1) the concentration of L-NO-Arg is insufficient to completely inhibit NOS, and 2) in this particular system, NO is not a major factor in the control of contractility. Therefore, the difference in force of contraction in control and haemin-treated hearts may be too small to demonstrate that L-NO-Arg exerts any significant effect. However, it is important to note that NO may play a role in the control of cardiac rhythm as 3 of the total of 8 hearts pre-treated with haemin and perfused with L-NO-Arg exhibited complete VF within 5-10 min of exposure to L-NO-Arg (Data not shown). However, NO release does not appear to affect the heart rate in the pre-ischaemic rat heart in either treatment group. This is in contrast to the results of a study by Kojda et al., (1997) where both endogenous and exogenous NO were

shown to alter heart rate; thus, for example, the use of L-NO-Arg decreased heart rate in their experiments.

Inhibition of COX with indomethacin did not significantly inhibit the vasodilator effect induced by haemin pre-treatment or alter the CPP in control hearts. This is in agreement with a number of studies, where there was little evidence for the role of prostaglandins in the regulation of coronary blood flow in the normal myocardium under physiological conditions (Harlan et al., 1978, Altman et al., 1992). Consequently, these data suggest that COX products, primarily the vasodilator PGI_2, are not involved in the CPP response induced by haemin pre-treatment. This is in opposition to a study by Leffler et al., (2001) where prostanoids and NO have been shown to contribute to CO-induced cerebrovascular dilation in pig pial arteries, although in this system, the interaction of COX products was determined after direct exposure to CO. In addition, as COX activity is dependent upon the binding of haem for regulation of its catalytic activity (Smith & Marnett, 1991), it could be envisaged that the increased expression of HO-1 may reduce cellular haem concentrations and influence the activity of COX (Maines, 1997), a response that could occur during the 24 hour exposure time to haemin prior to death. Furthermore, the anticipated increase in free iron release may affect COX activity. For example, cardiomyocytes exposed to iron loading demonstrate an increase in COX activity and COX-2 expression, which is accompanied by arachidonic acid release and by increased eicosanoid production (Qui & Quilley, 1999). Haider et al., (2002) confirm this in a study where COX expression and the release of prostaglandins are inhibited in cells transfected with the human HO-1 gene. Thus, the reduction in CPP appears to involve the coordinated actions of NO and CO, but not PGI_2. COX inhibition significantly increased developed tension in haemin pre-treated hearts, although the inhibition of COX in control hearts did not affect contractility. Heart rate was significantly reduced in haemin-treated hearts after COX inhibition. The combination of an increase in contractility and a decrease in heart rate in haemin- and indomethacin-treated hearts suggests that a similar mechanism is involved in both responses. This can be explained by the negative staircase effect, where an increase in heart rate induces a decrease in the force of contraction in the rat heart (Kelly & Hoffmann, 1960). Therefore, as a number of

COX products have been implicated in the control of heart rate, it is possible that COX inhibition may primarily affect heart rate. For example, PGI_2 has been shown either to increase or to decrease heart rate, as both tachycardia and bradycardia can be produced after i.v. injections of PGI_2 depending upon the basal heart rate (i.e. when the heart rate was high PGI_2 produced a bradycardia (Chiavarelli et al., 1982)). COX products have been demonstrated to produce various effects on cardiac tissue. For example, i.v. administration of PGI_2 can produce bradycardia (Chapple et al., 1980), or else it can activate 'C'-fibres to induce a reflex bradycardia (Baker et al., 1979). Furthermore, PGE_2 can induce tachycardia (Chapple et al., 1980).

6.3 The effect of haemin pre-treatment on the recovery of cardiac function after I/R.

6.3.1 The recovery of cardiac function in the constant-flow perfused rat heart.

Haemin pre-treatment induced a dose-related increase in recovery of contractility after 20 min I/R, with 75 μmol/kg haemin significantly increasing recovery. This finding is in agreement with the original study by Clark et al., (2000), the source of the dose of haemin used throughout the project. It is important to note that the conditions of perfusion were slightly different, as there was a longer ischaemic period of 30 min, higher constant flow rate of 15 ml/min and a higher K^+ concentration of the Krebs solution (5.9 mM K^+). Therefore, the common factors in the recovery of the heart tissue from I/R are the pre-treatment with 75 μmol/kg haemin for 24 hours, and the use of constant-flow perfusion. In addition, a recent paper has shown that the cardiac-specific expression of HO-1 has been shown to reduce cardiac dysfunction and cardiomyocyte apoptosis after ischaemia (Vulapulli et al., 2002). These data provide evidence that the protective effect of haemin during reperfusion is due to a directly beneficial action on the cardiac myocytes; this was independent of any changes in coronary flow because it was achieved using a constant flow perfusion system, and therefore there could be no changes in the supply of substrates, or in the washout of damaging substances.

Furthermore, haemin pre-treatment with 75 μmol/kg haemin significantly increased tissue bilirubin levels. The direct correlation between the level of bilirubin and the associated recovery of cardiac function makes it a prime candidate for involvement in this protective effect, as bilirubin is an anti-oxidant (Maines, 1997), and has previously been shown to be protective in the treatment of I/R when given exogenously (Clark et al., 2000). The protective role of bilirubin may include the removal of ROS that can be responsible for the tissue damage associated with I/R (Granger & Forthuis, 1995).

The protective effect of haemin pre-treatment does not appear to be related to a reduction in Ca^{2+}-overload as there is no significant effect on basal tension, although the combination of haemin and SnPP significantly reduced basal tension compared with haemin alone. This latter effect may have occurred as a result of increased oxidative stress due to the ROS-generating capacities of haemin and SnPP, in combination with the already increased stress levels induced by I/R (Schmitt et al., 1993, Ohse et al., 2001). Furthermore, haemin has been shown to inhibit the activity of the Na^{+}/K^{+} pump (Yasuhara et al., 1991), an important mechanism involved in the eventual induction of Ca^{2+} overload (Allen & Xiao, 2000).

SnPP did not significantly reduce the recovery of cardiac function in the presence of haemin pre-treatment, suggesting either that HO inhibition is not complete, or that other factors may be involved. The former suggestion implies that the dose of SnPP administered may not be high enough to sufficiently inhibit HO-1, but the dose selected was taken from Clark et al., (2000), where treatment with 40 μmol/kg SnPP for one hour abolished the protective effect of haemin so this seems unlikely. Alternatively, SnPP could be responsible for further effects contributing to I/R recovery; for example, metalloporpyrins have been described as peroxynitrite scavengers (Crow, 2000). It is important to note that the direct effect of SnPP on these parameters during reperfusion is dependent upon either the amount of circulating SnPP (which may be too low to produce such an effect), or the inhibition/stimulation of enzymes during the exposure to SnPP prior to the animals death. Incomplete inhibition of the improved recovery of

contractility induced by haemin pre-treatment may occur as a result of insufficient inhibition of bilirubin production. These data are also an indication of HO activity and suggest that HO activity is not completely inhibited.

The improved recovery of contractility is accompanied by a significant reduction in CPP during reperfusion, which is in agreement with the pre-ischaemic data, where a vasodilator response to haemin was also observed. This confirms the involvement of a vasodilatory mediator or the decreased release of a vasoconstrictor agent that is not dependent on ischaemic insult, in the response to haemin pre-treatment. As suggested in the pre-ischaemic heart, the most likely mediator is the vasodilator CO. The increase in HO activity (as measured by bilirubin production) suggests that CO production would be expected to be upregulated. However, in view of the fact that coronary flow was constant at 10 ml/min during the course of the experiment, this vasodilatation would not improve substrate supply to, or the washout of waste products, from the heart. Therefore, it is unlikely that change in vascular tone contributed to the beneficial effect on LVDT, although in an in vivo situation, where flow could change, this vasodilatation would be expected to have additional beneficial effects. The vasodilation produced by CO could be involved in an increase in tissue reperfusion; although the flow rate remains constant, the reduction in CPP may aid the prevention of tissue damage produced by the "garden-hose" hypothesis (Arnold et al., 1968, Poche et al., 1971). This phenomenon is described as the distention of coronary vessels that results in an increase in cardiac sarcomere length. Hence, in a system where CPP is increased, the force of contraction might be expected to increase. Therefore, vasodilation leads to not only a decrease in the "garden-hose effect" but also a decrease in energy consumption. Furthermore, peripheral vasodilation would also lead to a decreased load on the heart, and may increase flow to the deeper subendocardium. Thus, these data suggest that a reduction in CPP induced by haemin-pre-treatment could further increase recovery of cardiac function after I/R in our experiments. However, these data are not in agreement with Clark et al., (2000), as 75 μmol/kg haemin pre-treatment did not induce any significant change in CPP after I/R. In fact, part of the protective response to haemin was postulated to involve the maintenance

of CPP at pre-ischaemic levels. In an earlier study in our lab, hearts perfused at the higher K^+ concentration of 5.9 mM, demonstrated no significant reduction in CPP in the pre-ischaemic rat heart perfused at 10 ml/min (Data not shown). This may suggest that, under the conditions described by Clark et al., (2000), the maintenance of CPP may be due to the use of a Krebs solution containing 5.9 mM K^+.

SnPP did not significantly abolish the dilator response to haemin during reperfusion. It is possible that this occurred because HO inhibition is not complete, and that the response to haemin could still be mediated by HO expression. SnPP may also inhibit the response to HO-1 expression and CO release by the inhibition of guanyl cyclase (Grundemar & Ny, 1997). This could prevent CO from initiating both the activation of GC, and also the accompanying vasodilation. Furthermore, as discussed in the case of the pre-ischaemic heart, haemin pre-treatment has been shown to increase NOS activity (Suzuki et al., 1995), thus suggesting that other vasoactive mediators may be released.

6.3.2 The effect of haemin pre-treatment on recovery of cardiac function in rat hearts perfused at a constant-pressure of 70 mmHg.

After ischaemia the vasoconstrictor effect observed in the haemin-treated pre-ischaemic heart was abolished. It is possible that vasodilator mediators released during the reactive hyperemia response to I/R may serve to counteract the vasoconstrictor effect of haemin. However, haemin and SnPP produced a significant vasoconstrictor effect compared with control and SnPP. This suggests that haemin and SnPP are having a non-specific effect on the CFR during reperfusion as neither haemin or SnPP had any effect when acting alone. An example of a non-specific effect of SnPP is the inhibition of NO-mediated responses in the rat aorta, and this could affect the vascular responsiveness to NO (Vreman et al., 2000). Therefore, as haemin pre-treatment may reduce the responsiveness of guanyl cyclase to NO (Imai et al., 2001), this effect could be potentiated in combination with SnPP.

Recovery of contractility was marked (~80 %) in both haemin-treated and control hearts, suggesting that the method of perfusion used may increase recovery (in

comparison, the recovery observed in control hearts perfused at constant-flow was ~15 %). In the constant-flow model, we postulated that the reduction in CPP could aid recovery from I/R due to inhibition of the "garden-hose" effect. A study by Takeo et al., (1995) has demonstrated that reperfusion at reduced flow rates (over a range of 0.9-8 ml/min) can enhance the recovery of cardiac contractility post-ischaemia in comparison to a normal flow rate of 9 ml/min. Protection at low CFR was suggested to be due to a reduction in ischaemia-induced Ca^{2+} and Na^{+} overload, and was not induced by changes in either ROS generation or rate of Ca^{2+} delivery. This is compatible with our suggested reduction in the garden hose effect, which could cause cardiac myocytes to stretch, and thereby cause them to contract more forcefully (via the Starling effect), resulting in an increase in energy use. However, as there is no significant difference in CFR post-ischaemia, it is unlikely that a reduced flow rate pre-ischaemia is responsible for the high levels of recovery.

The reduction in recovery from I/R in SnPP-treated hearts may be related to an effect observed in the pre-ischaemic heart, where increased ROS generation potentiated the cellular damage associated with I/R. This could also be linked to an increase in Ca^{2+} overload, as the destructive effect of Ca^{2+} overload is produced by regulation of a number of Ca^{2+}-regulated enzymes such as phospholipases and degradative enzymes.

In the constant-pressure model, haemin pre-treatment did not significantly increase tissue bilirubin levels, which suggests that haemin pre-treatment did not confer any protection from I/R, due to the absence of an accompanying increase in bilirubin levels. This is in opposition with the data recorded in the constant-flow model, and also that observed by Clark et al., (2000), where the protection from I/R by haemin pre-treatment is reported to be due to bilirubin release. Furthermore, the amount of bilirubin measured in constant-pressure perfused hearts was found to be similar to that detected in control hearts perfused at constant flow, which were associated with a lower level of recovery. Under conditions of constant flow, bilirubin levels were increased 2-fold in haemin-treated hearts. SnPP reduced the recovery from I/R but increased bilirubin levels, in the presence of haemin. The increase in bilirubin production may be due to an

increase in the availability of the substrate haem. Hence, it appears that levels of bilirubin do not correlate with increased recovery from I/R in this model of perfusion. The increased level of bilirubin may actually be detrimental to the cells, as reported by Suttner and Dennery (1999), where high levels of HO-1 expression actually increased cell death.

6.3.3 The contribution of NO and COX products on the recovery from I/R of haemin treated hearts.

The release of NO as a result of haemin pre-treatment does not appear to be involved in the recovery of cardiac function after I/R. There has been some controversy about the positive or negative role of NO in I/R, although of the studies involved around 73 % conclude that NO has a protective role in both partial and global ischaemia models (Bolli, 2001). Of these studies, Wesselcouch et al., (1995) and Ferdinandy et al., (1997) have reported that endogenous NO has little effect on the recovery from ischaemic insult. This is in agreement with the data presented here, as control hearts exposed to NOS inhibition did not demonstrate a significant reduction on recovery of cardiac function. A reduction in Ca^{2+} overload was observed in the presence of L-NO-Arg, but this had little influence on contractility under these conditions. Furthermore, this was accompanied by a lack of influence on tissue bilirubin levels, suggesting that the degree of protection that remains after NOS inhibition may be solely due to the maintenance of bilirubin levels. NO release is involved in the vasodilator response to haemin treatment post-ischaemia, which is in agreement with the effect observed in the pre-ischaemic heart, and also a study by Wesselcouch et al., (1995), where the inhibition of NO synthesis produced coronary vasoconstriction, although this was accompanied by cardiac depression. However, as there was little change in control hearts, it seems that this effect was initiated by pre-treatment with haemin.

After I/R, COX inhibition significantly reduced the recovery of cardiac function induced by haemin pre-treatment. These data implicate the involvement of COX products in the recovery of cardiac function induced by haemin pre-treatment

and are in agreement with our data from the pre-ischaemic heart, where COX products appear to increase the force of contraction. A study by Morbert & Becker, (1998) demonstrated that COX inhibition exacerbated the recovery of cardiac function after ischaemia. This is also shown in the guinea pig heart, where aspirin-induced inhibition of COX aggravated post-ischaemic cardiac dysfunction (Heindl & Becker, 2001). The exogenous administration of a PGI_2 analogue has been shown to increase recovery of cardiac function after I/R (Maulik et al., 1997). Hence, COX inhibition may significantly reduce recovery of cardiac function due to inhibition of PGI_2 production. In addition, the reduction in the improved recovery of contractility induced by haemin pre-treatment was not related to an effect on Ca^{2+} overload, as indicated by the lack of effect on basal tension.

Indomethacin reduced the CPP response to haemin pre-treatment. This is in opposition to data observed in the pre-ischaemic heart, where there was no significant effect on haemin-induced vasodilation. This may be related to the fact that previous studies have demonstrated that reperfusion is a potent stimulus for PG synthesis (Karmazyn, 1986). COX products have also been implicated in the induction of cardiac reactive hyperaemia after ischaemia (Gryglewski et al., 1996). Moreover, in a rat model of low-flow ischaemia, COX inhibition using indomethacin prevented the preservation of vasodilation after ischaemia, which is associated with the protection of endothelial function (Bouchard & Lamontagne, 1999). Furthermore, COX inhibition can lead to substrate diversion to the lipoxygenase pathway, resulting in the production of leukotrienes (LT), which are potent vasoconstrictors (Maulik et al., 1997).

The inhibition of COX in haemin-treated hearts significantly increased tissue bilirubin production. Therefore, COX inhibition appears to influence the activity of HO-mediated haem breakdown. As COX activity is dependent upon the binding of haem for the regulation of its catalytic activity (Smith & Marnett, 1991), it is possible that inhibition of COX results in the increase in free haem and bilirubin production.

6.4 The effect of increasing the duration of the ischaemic period on the recovery of cardiac function.

6.4.1 The effect of 30 min ischaemic insult on recovery of cardiac function in the constant –flow-perfused rat heart.

The recovery of cardiac function post 30 min I/R was significantly reduced in both control and haemin-treated hearts. This data is not in agreement with the study by Clark et al., (2000), where rats hearts pre-treated with 75 μmol/kg haemin (24hours) were exposed to a 30 min ischaemic period; this may be because of slight experimental differences between the 2 studies. Interestingly the lack of protection was not accompanied by a reduction in tissue bilirubin levels. The increase in ischaemic period has probably produced an irreversible damage compared to a 20 min ischaemic period (Taegtmayer et al., 1997). Furthermore, as bilirubin levels are not significantly affected, we postulate that the level of tissue bilirubin is insufficient to prevent the increased tissue damage caused by an additional 10 min of ischaemia, and therefore we conclude that the cells may already be damaged by ischaemia in this case. Thus, the limiting factor in determining the degree of recovery of cardiac function from I/R in this system is the length of ischaemic insult.

The eradication of the protective response from haemin pre-treatment is also accompanied by an absence of vasodilation during reperfusion. These data are in agreement with Clark et al., (2000), where little change in CPP above baseline was recorded in hearts treated with haemin. This response is likely to occur as a result of either increased vasoconstrictor release, decreased vasodilator release or diminished vascular responsiveness as a result of endothelial cell injury post ischaemia, or a combination of all. Therefore, the recovery of cardiac function after I/R under our experimental conditions appears to require a vasodilatory mediator, rather than an increase in tissue bilirubin production, after an ischaemic insult of 30 min.

6.4.2 The effect of increasing the duration of the ischaemic period on recovery of cardiac function in the constant-pressure perfused rat heart.

In control hearts subjected to 30 and 40 min ischaemia, there was a significant reduction in the reactive hyperemia response. Reactive hyperemia is a vasodilator response that occurs in response to I/R, and its removal may be caused be either a decrease in vascular responsiveness, a decrease in release of vasodilators, or an increase in release of a vasoconstrictor. For example, adenosine, NO, and K_{ATP} channels have all been implicated in reactive hyperemia of the guinea-pig heart (Kingsbury et al., 2001). A study by Kirkeboen et al., (1994), has indicated that endothelium-derived NO may be involved in reactive hyperemia after a short duration of coronary no-flow ischaemia but that it is absent or reduced in reactive hyperemia after a prolonged ischaemia. This is also true of K_{ATP} channel activity in the rat heart (Shinoda et al., 1997). This evidence suggests that reactive hyperemia is abolished due to increases in ischaemic insult. An alternative suggestion for the reduction in CFR may be due to oedema formation, which can cause cell swelling to occlude vessels, another mechanism of the "no-reflow" phenomenon (Korthuis et al, 1994). Our data suggest that there is a greater vasoconstrictor effect in haemin – treated hearts after increased ischaemic insult. It is possible that the balance of vasoactive mediators may become disrupted after extended ischaemia, so that the removal of vasodilator mediators such as NO and adenosine (Kingsbury et al., 2001, Kirkeboen et al., 1994) enhances the effect of HO-1 induction.

The increased ischaemic insult significantly reduced recovery of contractility after I/R to a similar extent indicating that there is only a very small therapeutic window in which the hearts will demonstrate almost complete recovery from I/R in this model. This is in agreement with the current understanding that as the ischaemic insult increases, recovery from I/R becomes impaired due to the change from reversible damage after 20 min ischaemia to irreversible damage after 30 min+ (Taegtmayer et al., 1997). Haemin pre-treatment did not confer any added protection from prolonged I/R injury. This result was observed in both the constant-flow and the constant-pressure-perfused rat heart. This is in opposition with previously recorded data, where haemin pre-treatment or HO-1 overexpression significantly increased recovery after a 30 min ischaemic insult in

the constant-pressure-perfused heart (Yet et al., 2001), but it should be noted that there were slight experimental differences between the 2 studies, such as higher flow rates and different methods of HO-1 induction and perfusion. It is difficult to comprehensively determine the true effect of HO-1 expression in the in vitro perfused heart due to the diverse methods used to investigate such effects.

The reduced recovery of contractility is accompanied by a significant increase in Ca^{2+} overload. As it can be postulated that acidosis may be potentiated during the prolonged ischaemic insult as a result of increased ATP depletion (Bolli et al., 1990), it is likely that increases in intracellular Ca^{2+} upon reperfusion may be enhanced.

Tissue bilirubin levels were increased in a time-dependent manner in control and haemin-treated hearts. The increased bilirubin levels do not confer protection from I/R, but it is conceivable that this result occurs in response to the increasing oxidative stress seen after increased ischaemic insult, as HO-1 activity can be increased in the presence of ROS generation (Vile et al., 1994, Panchenko et al., 2000). It is also possible that increased tissue damage and cellular apoptosis may increase free haem levels (Maines, 1997) and thus increase substrate levels for the production of bilirubin. The increased tissue bilirubin levels may be present as a result of a decreased washout due to the vasoconstrictor effects observed during reperfusion. Furthermore, in cardiomyocytes exposed to hypoxia and reoxygenation, haem availability is the rate-limiting factor involved in the protection conferred by HO-1 induction (Foresti et al., 2001).

6.5. Does perfusing the rat heart at 130mmHg affect the recovery of cardiac function after I/R?

In control hearts perfused at a constant flow of 10 ml/min the initial CPP averaged 130 mmHg compared with 70-90 mmHg in haemin-treated hearts. Therefore, the effect of perfusion at a constant pressure of 130 mmHg was investigated to determine the influence of the pressure level on recovery from I/R and vascular responsiveness. Haemin pre-treatment produced a vasoconstrictor response, indicating that this effect is not dependent on the pressure of system. Furthermore, the CFRs of control and haemin-treated hearts were approximately

double those recorded previously in hearts perfused at 70 mmHg, indicating that autoregulatory mechanisms are not present in this system. At 130 mmHg, contractility is decreased and heart rate is increased. The increase in heart rate may induce a reduction in contractility in the phenomenon known as the "negative staircase" (Kelly & Hoffmann, 1960), where an increase in heart rate produces a decrease in contractility or vice versa.

Upon reperfusion, haemin pre-treatment produced a vasoconstrictor response compared with control; this is in agreement with pre-ischaemic data. There appears to be an inhibitory effect (which is not present at 70 mmHg) on reactive hyperemia in haemin-treated hearts. This suggests that the vasoconstrictor effect post-ischaemia is dependent upon the basal pressure. In a study by Wagner et al., (1997), the expression of HO-1 has been shown to be regulated by haemodynamic forces in vascular smooth muscles. For example, the passage of blood through the cardiovascular system induces endothelial cell elongation in response to shear stress via cytoskeletal rearrangement and the secretion of various bioactive molecules. However, the time course of this experiment is not long enough to demonstrate an increase in HO-1 expression, although an increase in HO activity could occur. For example, a study by Carraway et al., (2000) has indicated that the pattern of HO-1 expression does not correspond with the pattern of HO activity in the chronic hypoxic lung. Therefore, this may suggest that there may be a post-translational mechanism involved in increasing HO activity after expression.

Haemin conferred significant protection on the recovery of contractility at 130 mmHg compared with control. However, the degree of recovery of contractility was significantly reduced at 130 mmHg compared with that previously recorded at 70 mmHg. This suggests that the conservation of cardiac function by haemin-induced HO-1 may be pressure-sensitive under constant-pressure perfusion conditions. Furthermore, the reduction in recovery of contractility is not connected to an increase in intracellular Ca^{2+} overload.

Haemin pre-treatment increased bilirubin levels compared with control. The recovery of contractility in haemin-treated hearts appears to involve an increase

in bilirubin production. It is possible that this effect may be produced by a direct effect on muscle contraction. For instance, Samb et al., (2001) reported that HO and bilirubin control smooth muscle reactivity to ROS-induced phosphorylation of the myosin light chain in the guinea pig trachea.

6.6 The effect of haemin-induced HO-1 expression on anaerobic glycolysis in the post-ischaemic rat heart

The main aim for the investigation of the effect of haemin pre-treatment on anaerobic glycolysis was to determine whether the protective effect of haemin on I/R was related to an increase in ATP production during ischaemia, or a reduction in the build-up of detrimental metabolites such as lactate and H^+. For example, in the preconditioned dog heart, it is postulated that preconditioning protects the heart by reducing the metabolic requirements of the ischaemic myocardium, and therefore preserving ATP or limiting the production of catabolite accumulation (such as lactate) (Murry et al., 1990).

Treatment with haemin did not significantly affect lactate release compared with control at constant-flow and constant-pressure, indicating that the protective effect of haemin post I/R is not due to an increase in anaerobic ATP production via glycolysis. The involvement of the activation of cGMP in the regulation of glucose metabolism has been previously demonstrated by the use of cGMP analogues to decrease glucose uptake and glycolytic flux (Depre et al., 1999). Therefore, we questioned whether CO release in haemin-treated hearts may influence glucose metabolism due to its action on cGMP. Our results suggest that this is not the case. Furthermore, a recent study by Chang et al., (2003), has suggested that HO-1 gene activation can occur as a result of glucose deprivation, via a mechanism which involves the generation of free radicals. However, the reduction in recovery of hearts treated with a combination of haemin + SnPP may be related to an accumulation of glycolytic metabolites such as lactate and H^+ (Murry et al., 1990). Alternatively, any increase in lactate would be expected to be accompanied by an increase in ATP production, a protective response to I/R. Inhibition of NO release may significantly reduce ATP production during ischaemia and promote the accumulation of toxic metabolites such as lactate.

Furthermore, this effect appears to occur regardless of haemin pre-treatment and is exclusively related to the release of NO. This is in agreement with a study by Depre et al., (1995), where NO donors were found to increase glucose metabolism in the ischaemic heart. The negative value recorded during the fifth minute of reperfusion in the presence of L-NO-Arg suggests that some lactate may have been pumped back into the cardiac tissue. COX products do not appear to influence the production of ATP from anaerobic glycolysis in the ischaemic rat heart. This suggests that the reduction in recovery of cardiac function in the presence of indomethacin is not produced as a result of decreased ATP production from anaerobic glycolysis or increased metabolite accumulation. Finally, after increasing the duration of the ischaemic period to 30 min at constant-flow, total lactate release is increased in control hearts, compared with a decrease in control hearts perfused at constant-pressure. These contradicting data suggest that by increasing the ischaemic period in control hearts perfused at constant-flow, a prolonged period of anaerobic glycolysis is brought about as indicated by the increase in lactate. However, as there is no significant difference in the total lactate release at constant-pressure, it is possible that the reduction observed during the first 2 min may occur as a result of the significant vasoconstrictor effect observed. In contrast, as there is no difference in haemin-treated hearts, this suggests that, as the ischaemic period increases, anaerobic glycolysis or the increase of glucose uptake for glycogen formation may be inhibited. In theory, there should be the same amount of substrate available in both control and haemin-treated hearts.

Thus, at a constant pressure of 130 mmHg, the improved recovery of contractility after I/R is not related to an effect on anaerobic glycolysis or ATP production. However, according to Taegtmayer et al., (1997) tissue ATP content does not correlate with contractile function. This seems also to be true for haemin pre-treatment, as it induces increased recovery but no difference in lactate release. The improvement of recovery in the presence of DFO is not assisted by a reduction in lactate production or washout.

6.7 What effect does haemin pre-treatment have on the release of nitrite ?

Nitrite release is decreased in haemin –treated hearts and increased in SnPP-treated hearts. Haemin pre-treatment may decrease NO production in the pre-ischaemic rat heart, thereby bringing about a vasoconstrictor effect. This corroborates with the data published by Imai et al., (2001) where vascular-specific expression of HO-1 produced an increase in blood pressure by suppressing the vasodilatory response to NO. Moreover, a study by Kostic et al., (1996), suggests that NO is involved in the control of basal coronary flow as indicated by a reduction in CFR after NOS inhibition. Therefore, the important role of NO under basal conditions indicates that increases in HO-1 expression can reduce NO release (Maines, 1997) and produce an overall constrictor effect. These data may suggest a role for HO-2 in the control of NO release, although this does not affect the overall CFR compared with control. At a constant pressure of 130 mmHg, haemin does not significantly affect the release of NO in the pre-ischaemic rat heart compared with control, implying that the vasoconstrictor effect does not involve an action on NO release. Rubanyi & Vanhoutte, (1986), have reported that the action of NO is potentiated as a result of an increased flow-induced mechanism. Thus, the constrictor effects of haemin pre-treatment are produced via different mechanisms at 70 and 130 mmHg.

Upon reperfusion at 70 mmHg or 130 mmHg, the release of nitrite is significantly reduced in haemin-treated hearts compared with control during the first 2 min of reperfusion. Inhibition of NOS has been shown to decrease the duration of the hyperemic response, but it is important to note that a reactive hyperemia is still present, thus excluding NO as the main mediator of reactive hyperemia (Pohl & Cor de Wit, 1999). SnPP increased nitrite release over the initial 5 min of reperfusion. This indicates that a non-specific effect of SnPP is taking place, as there is no significant difference between treatment with SnPP alone or in the presence of haemin. This data is in opposition with a previous observation that metalloporphyrins reduce NO-mediated responses in the rat aorta (Vreman et al., 2000). However, a report by Chakder et al., (1996), suggests that the metalloporphyrin ZnPP can stimulate NOS activity in the rabbit internal anal sphincter, albeit at very high non-specific doses.

6.8 Does the removal of free iron using the iron chelator desferrioxamine affect the pre-ischaemic heart or increase the recovery from I/R?

DFO reversed the vasoconstrictor effect of haemin pre-treatment, suggesting that there is an element of iron involvement in the control of CFR perfused at constant pressure. However, this may occur due to a non-specific effect of DFO. Studies have reported that DFO can act as a direct scavenger of hydroxyl radicals (Hoe et al., 1982). DFO may also influence the recovery of coronary flow after ischaemic insult (Ambrosio et al., 1987); this effect does not appear to be directly related to DFO itself as other iron chelators such as desferriexochelin have been proven to increase the recovery of portal venous blood flow, decreased hepatocyte injury and reduced oxidative stress levels in the I/R liver model (Amersi et al., 2001).

Furthermore, iron released during haem degradation could further inhibit NOS mRNA synthesis by inhibiting nuclear transcription (Weiss et al., 1994). However, ROS have been implicated in the production of a vasodilatory effect (Shattock et al., 1982). DFO reduced the force of contractility in haemin-treated hearts, implying that free iron may have a significant influence in the regulation of the force of contraction in haemin-treated hearts. This is surprising as the converse might be expected, as the presence of ROS produced by increased iron levels have been shown to cause contractile dysfunction (Farber et al., 1988). It is possible that this effect may be caused by continued perfusion with DFO throughout the duration of the experiment. Other non-specific actions of DFO such as the removal of hydroxyl ions could also affect contractility (Hoe et al., 1982). However, the addition of DFO did not affect heart rate.

A 30 min ischaemic insult provided a large enough therapeutic window to demonstrate the potential impact of DFO treatment on the recovery from I/R. DFO treatment significantly increased recovery from I/R in both control and haemin-treated hearts to a similar extent. The involvement of free iron has been demonstrated in the pathogenesis of I/R (Berenshtein et al., 1997), and may occur as a result of the rapid release of iron from ferritin under anaerobic conditions (Bolli et al., 1990), or after attack by superoxide radicals (Antonius et

al., 1988). Ambrosio et al., (1987) have demonstrated a significant increase in recovery of myocardial function and energy metabolism after global ischaemia when DFO is added upon reperfusion. DFO has been proven to reduce the occurrence of reperfusion-induced VF in rat hearts (Bernier et al., 1986). Myocardial "stunning", described as the prolonged reduction in contractility in the reperfused myocardium after reversible ischaemic insult, has also been inhibited by the presence of DFO, and this has been suggested to be due to the reduction of iron-catalyzed ROS production (Bolli et al., 1990). Furthermore, the improved recovery from I/R in haemin-treated hearts in the presence of DFO may be related to a reduction in intracellular Ca^{2+} overload.

An increase in CFR was associated with the DFO-induced improvement in recovery of contractile function in control hearts. This is in agreement with a study by Badylak et al., (1987), which indicated that DFO reduced the increased vascular resistance associated with reperfusion. Furthermore, DFO has been shown to influence the recovery of coronary flow after ischaemic insult (Ambrosio et al., 1987). Further studies have demonstrated an improved recovery of coronary flow from DFO treatment after hypothermic ischaemia, but this was not accompanied by a beneficial effect on recovery of contractility (Myers et al., 1986). The removal of the free iron generated by ischaemia may aid a protective response by increasing CFR and thereby preventing the onset of the events described in the garden "hose" hypothesis (Arnold et al., 1968, Poche et al., 1971). The beneficial effects of DFO are not restricted to CFR, as there is no significant increase in the CFR of haemin-treated hearts. However, the anti-apoptotic effect of HO-1 appears to be related to an ability to increase cellular iron efflux via the upregulation of an uncharacterized iron pump (Brouard et al., 2000). Alternatively, the upregulation of HO-1 activity and associated increase in free iron release also increases the expression of ferritin (Vile et al., 1994). These factors could explain why ischaemic insult exerts little effect on the CFR of haemin-treated hearts exposed to DFO. However, it is possible that the iron-DFO complex that is produced can still facilitate an increase in HO-1 expression, and thereby bring about an increase in bilirubin production.

6.9 Is the vasoconstrictor effect of haemin-induced HO-1 expression caused by an effect on the autoregulation mechanisms of the rat heart?

Coronary autoregulation is described as the capacity to maintain a stable blood flow supply within the coronary circulation in the face of wide-ranging change in perfusion pressure. The heart achieves this by initiating vascular myogenic changes in response to alterations in pressure, and by a number of metabolic changes. For example, increased pressure results in an initial increase in flow, which enables the washout of metabolites leading to vasoconstriction and eventually normalized flow (Pohl & Cor de Wit, 1999). It is possible that increased expression of HO-1 and CO production may interact with these vasoactive mediators and autoregulation mechanisms.

Haemin pre-treatment produced a vasoconstrictor effect at 100, 80 and 60 mmHg, providing further evidence that haemin has a vasoconstrictor effect at various pressure levels. However, haemin does not significantly reduce CFR at 135 and 120 mmHg in this system. Interestingly, the combination of haemin and SnPP produced VF after the initial pressure change in 3 of the 5 tissues examined. It is difficult to explain the mechanism behind such responses, although it is possible that there was an interaction between haemin + SnPP that proved detrimental at a higher pressure level. This could not be confirmed by referral to previous experiments at 130 mmHg, as the effect of SnPP had not been investigated in these cases. SnPP treatment produced a CFR similar to haemin-treated hearts. This effect could be another demonstration of the dual mechanism of action thought to be produced by various metalloporphyrins, in particular SnPP (Maines, 1997). Alternatively, the increased ROS generation produced by SnPP (Ohse et al., 2001) may act to reduce the half-life of NO, and thereby bring about an overall constrictor reponse.

The CFR was measured over an initial period of 2 min after the pressure change in each treatment group. After the initial pressure change to 135 mmHg, there was a significant reduction in the CFR of tissues treated with haemin and SnPP compared with control. This low flow rate at a relatively high pressure may suggest a reason for the production of VF relatively soon after the pressure

change. The overall conclusion of this experiment is that there is no clear evidence of any autoregulation mechanisms in both control and haemin-treated hearts as demonstrated by the gradual reduction in CFR as the pressure level is reduced. This is in agreement with the data measured at 130 mmHg, where the CFR doubled compared with hearts perfused at 70 mmHg. If autoregulation mechanisms were active, the CFR would remain the same regardless of the perfusion pressure.

In the presence of NO, there is a prominent autoregulation effect in the heart, although Pohl & Cor de Wit, (1999) have suggested that NO can actually attenuate autoregulation during myogenic autoregulation. NO has been shown to be one of the primary mediators involved in metabolic control of coronary autoregulation, although the actual mechanisms involved in autoregulation are likely to be more complex. Furthermore, Jakovljevic et al., (1999) have reported an interaction between the L-arginine-NO system and COX products in the regulation of coronary autoregulation. Thus, it is possible that as there has been previous evidence that HO-1-derived CO can also interact with NO and COX products, there may some sort of interaction under these conditions. It has been established that in a situation where there is a permanent effect on either tissue activity or metabolic changes, autoregulation mechanisms can be reset to new levels. As the use of SnPP continually reduced CFR throughout the experiment, it can be postulated that endogenous CO production may be important in autoregulation mechanisms. However, the data observed in haemin-treated hearts also produced a conflicting effect where a negative role was implicated for increased HO-1 expression in the autoregulation response. Alternatively, this may be a first example of a possible opposing effect from HO-1 and HO-2 in the control of vasoreactivity.

6.10 The effect of haemin pre-treatment on vascular tone in other vascular beds.

Investigation of haemin pre-treatment in the perfused mesentery and kidney provided an opportunity to study the effect of HO-1 in other vascular beds. (However, immunoblot analysis was not carried out in these tissues to determine whether HO-1 expression was significantly increased. Therefore, any effect of HO-1 on vascular responsiveness in haemin-treated preparations must be considered to be purely speculative.)

6.10.1 How does haemin-induced HO-1 expression influence vascular responsiveness in the perfused mesentery ?

In the perfused mesentery, haemin pre-treatment produced a small increase in the initial perfusion pressure although this was not statistically significant. In contrast, haemin did attenuate the vasoconstrictor effect of PE (10 μM), but did not affect the response to bolus doses of NA. In addition, in haemin pre-treated mesenteric vascular beds, it was very difficult to maintain a high enough perfusion pressure to investigate the activity of vasoconstrictor agents. Therefore, it is possible that if haemin pre-treatment induces HO-1 expression, the prolonged increase in basal tone induced by PE may be influenced by CO release, resulting in attenuation of the PE-induced perfusion pressure. For example, in a model of hypertension involving the chronic perfusion of angiotensin II, HO-1 expression has been reported to increase in the rat aorta and heart as a result of haemodynamic / mechanical stress and pressure overload (Ishizaka et al., 1997, Ishizaka et al., 2000).

The responses to ACh were very small in haemin-treated tissues compared with control, which may be due to the influence of HO-1-induced CO release. HO-1 has been shown to protect against vascular constriction (Duckers, 2001). Hence, it is possible that haemin reduces the response to ACh through a direct effect on M_3 receptors. The most likely effect is the potentiation of relaxation by NO, the effector molecule responsible for vasodilation via the M_3 receptor. The role of

NO in response to haemin-induced HO-1 expression has been an important theme throughout this study.

In contrast, haemin does not affect the response to histamine apart from at the highest dose. Therefore, it is possible that this is in response to the force of vasoconstriction induced by a high dose of histamine, and may not be specifically linked to a histamine receptor-mediated event. The second messenger system involved in generating the response to histamine H1 receptor activation produces an overall increase in intracellular Ca^{2+} levels. Therefore, an increase in HO-1 expression may not influence intracellular Ca^{2+} levels at lower doses. Alternatively, as CO has been shown to inhibit the activity of the K_{Ca} channel (Wang & Wu, 1997), it is likely that the levels of intracellular Ca^{2+} may not be increased at levels where CO can exert an effect (apart from at the highest dose of 30 nmol histamine).

The effect of haemin-induced HO-1 expression on vasoconstriction in response to PE, ET-1, NA and Ang II was investigated. At the highest dose of PE, haemin treatment significantly reduced the vasoconstriction. This suggests that HO activity may be increased in response to an increase in the degree and duration of response to PE as haemodynamic stress is an inducer of HO activity (Wagner et al., 1997). However, there was no effect on the dose-response curves of vasoconstriction versus NA or Angiotensin II. Both haemin and SnPP (alone and in combination) reduced the duration of response to ET-1 (highest dose only). Therefore, haemin pre-treatment does not affect the mesenteric response to vasoconstrictors except after a prolonged exposure time. CO has recently been attributed as an endogenous endothelial-derived vasodilator in the mesenteric circulation (Naik et al., 2003), but its role in the response to vasoconstrictors is as yet undetermined.

6.10.2 How does haemin-induced HO-1 expression regulate vascular responsiveness in the perfused kidney ?

Many studies have recorded a HO-1-induced protective effect in the rat kidney. Haemin pre-treatment did not significantly affect basal perfusion pressure. This suggests either that upregulation of HO-1 expression does not influence basal pressure in the rat kidney perfused at constant-flow, or that the selected dose of haemin did not elevate HO-1 expression. However, a study by Wiesel et al., 2001, demonstrated that HO-1 gene removal did not significantly affect basal perfusion pressure in the mouse kidney. To investigate the effect of HO-1 expression on vasodilators, the basal tone was increased using 30 mM K^+; this K^+-induced increase in perfusion pressure was not affected by haemin. Therefore, HO-1 induction did not affect vasoconstriction induced by K^+. Kaide et al., (2001), also reported that the induction of HO-1 and CO release did not significantly affect the vasoconstrictor response to KCl in the kidney. This indicates that HO-1 does not minimize the KCl-induced increase in cytosolic Ca^{2+} in smooth muscle cells (Wang et al., 1998), and could indicate that even if HO-1 expression is increased, it is not active under these conditions.

The vasodilator dose-response curve obtained in response to histamine in the control and haemin-treated kidneys were almost exactly the same. Thus, the presumed induction of HO-1 does not modulate the vasodilator response to histamine. As histamine produces a vasodilation via activation of H_1 receptors and an increase in intracellular Ca^{2+}, this indicates that CO does not influence histamine-induced Ca^{2+}mobilization. In accordance with a study previously carried out in our lab (Laight, 1994), it was very difficult to achieve a vasodilator dose-response curve to ACh. The effect of haemin pre-treatment in response to vasoconstrictors was also determined in the perfused kidney. Dose-response curves of PE, ET-1 and SX6C were obtained in both control and haemin-treated kidneys. Haemin pre-treatment significantly reduced the peak height response to PE compared with control ($p<0.05$). A similar effect has previously been illustrated in the rat kidney, where HO-1 induction was found to reduce the sensitivity to PE; this was shown to be due to CO release acting on Kca channels (Kaide et al., 2001). In contrast, SnPP, which inhibited HO, produced a

significant decrease in duration of response to PE (0.3 to 10 nmol) in haemin-treated kidneys. In the study by Kaide et al., (2001), the inhibition of HO resulted in an increase in sensitivity to PE. This was not manifested as a change in the size of the response, but suggests that a HO-2 product may be important in the duration of response. For example, under normal conditions it is possible that HO-2 mediated CO release may be responsible for a slower return to baseline than as exhibited in the SnPP-treated tissues. These responses may be important in pathological situations, such as the chronic hypoxic rat kidney, where chronic hypoxia has been shown to increase HO-1 activity, and the resultant CO release is important in the production of a vasodilatory influence in the kidney (O'Donaughy & Walker, 2000).

Haemin pre-treatment attenuated the constriction effect of the highest dose of ET-1, and also reduced the duration of response at 30 and 100 pmol ET-1. The reduction of the peak height response to the highest doses of ET-1 may occur as a non-specific response to the highest dose. To further investigate this response, the effect of haemin treatment on the constrictor nature of the ET_B agonist SX6C was examined. The extent and duration of the response to SX6C was similar in both control and haemin-treated tissues. Therefore, the effect of haemin treatment on the response to ET-1 does not appear to involve an influence on the ET_B receptor.

CHAPTER 7:
CONCLUSION

Chapter 7: Conclusions

7.1 The opposing vascular effects of haemin pre-treatment in the constant-flow and constant-pressure-perfused rat hearts.

Throughout the course of the study, haemin has been used as an inducer of HO-1 expression. One of the most interesting observations to be made from the rat heart experiments has been the opposite vascular responses in the pre- and post-ischaemic rat hearts. In the constant-flow heart model, haemin-induced HO-1 expression was associated with a vasodilator effect, as indicated by a reduction in CPP, in the pre- and post-ischaemic rat heart. We postulate that this may be due to HO-1-mediated CO release. In contrast, in the constant-pressure-perfused rat heart, the same dose of haemin produced a reduction in CFR (which is indicative of a vasoconstrictor response) in the pre-ischaemic heart, but this was not so pronounced in the post-ischaemic heart. The causes of this vasoconstrictor effect are not clear, but they may involve an effect on NO generation/release. The two-perfusion systems used give an opportunity to study different parameters, and to investigate the control systems involved in the regulation of coronary flow or pressure. For example, the constant-flow perfusion system allows changes in contractility to occur which are independent of substrate delivery, as flow is maintained when heart rate is changed or regional ischaemia occurs (Sutherland & Hearse, 2000). We believe that due to the different perfusion techniques used, this is the first study indicating such a complex effect after HO-1 induction. The constant-pressure perfusion system is the more physiological of the two systems examined, as it represents the nearest approximation to the regulation of pressure in the human body.

Therefore, do these data indicate that HO-1 expression could produce a constrictor effect *in vivo*? The majority of the data previously obtained from *in vivo* situations suggests that induction of HO-1 causes a reduction in blood pressure, whereas there is only one report of increased blood pressure after cardiac-specific induction of HO-1 expression (Imai et al., 2001). Therefore, it would be premature to suggest that our data imply that HO-1 induction constitutes a detrimental effect in the whole body. For example, the experimental

systems from which both sets of data were recorded are cardiac-specific and do not take into consideration any the other systems involved in the regulation of vascular tone and blood pressure. Nevertheless, it is important to note that the coronary vasoconstrictor effect was also observed at the higher perfusion pressure of 130 mmHg, throughout the autoregulation experiments, and after an ischaemic insult of 30 min. The measurement of nitrite enabled the examination of the effect of HO-1 expression on NO release and suggested that an element of the constrictor response may involve a reduction of NO release. This is in contrast with the data observed in the constant-flow-perfused rat heart, where NOS inhibition indicated that NO release worked alongside HO-1 induction to bring about a dilator response to haemin pre-treatment. The interactions between NOS/NO and HO-1/CO have been well documented. Therefore, it is possible that, while NO may be involved in both responses, the method of perfusion and the regulatory mechanisms incorporated in the control of pressure/flow affect the nature of the involvement.

To further investigate this, the following experiments would be useful:

1. The investigation of the effects of the constant-flow and constant-pressure perfusion systems on specific HO-1 inhibition using oligodeoxynucleotides, or in a cardio-selective knockout system.
2. The use of cardiac-specific HO-1 up-regulation techniques to eliminate any non-specific actions of haemin treatment.
3. The investigation of the effects of HO-1 induction on the expression of NOS under each individual perfusion condition, and its relationship to the vascular responses observed.
4. The examination of the effects of various constant-pressure perfusion levels on HO activity, in relation to the data recorded at 130 mmHg.
5. The analysis of further doses of SnPP to determine whether complete inhibition of haemin-induced responses could be achieved.
6. The further exploration of the possible non-specific side effects of both haemin and SnPP in these systems.

7.2. The role of HO-1 induction in recovery from I/R in both the constant-flow- and constant-pressure-perfused rat heart.

The control of vascular dynamics may be important in regulating the degree of recovery from I/R; does the method of perfusion used in our experiments affect haemin-induced recovery after I/R? In the constant-flow-perfused heart, haemin pre-treatment increased contractile recovery following 20 min ischaemia. This was postulated to be due to the antioxidant properties of bilirubin, levels of which were significantly increased after haemin pre-treatment. In contrast, when hearts were perfused at constant-pressure following 20 min ischaemia, recovery was improved to about 80 % in the controls and haemin-treated hearts. Therefore, in this system, haemin-induced HO-1 expression did not increase protection compared with control, and similar levels of bilirubin were seen in both treatment groups, and as no changes were seen it did not seem relevant to investigate the influence of any further drugs. Further studies were performed using a longer ischaemic period of 30 min to establish if haemin could improve recovery from a more severe ischaemic insult. However, in the constant-flow and constant-pressure perfusion systems, there was a complete elimination of recovery from 30 min I/R in both control and haemin-treated hearts. This is not in agreement with the study by Clark et al., (2000), that also used a constant-flow system, and was used as reference point for the present study. Clark et al., (2000) reported a significant increase in recovery in haemin-treated hearts compared to control after 30 min ischaemia under constant-flow conditions of 15 ml/min, experimental conditions which are slightly different from ours. This discrepancy may have influenced the degree of recovery induced by haemin pre-treatment. In addition, other studies have indicated that HO-1 induction is protective after 30 min ischaemia (Yet et al., 2001). Although in this study, bilirubin levels in hearts perfused in constant-flow and constant-pressure systems were increased in response to increased HO-1 activity, these increases did not always confer protection of cardiac function. Thus, the mechanisms involved in the protective effect of HO-1 induction are more complex than a simple increase in bilirubin activity.

In the constant-pressure system, haemin-induced protection from I/R was observed at a constant pressure of 130 mmHg, suggesting that the protection produced by haemin-induced HO-1 expression in the constant-pressure-perfused rat heart is pressure-sensitive or flow-dependent. In conclusion, the induced recovery from I/R in the constant-pressure-perfused rat heart appears to be more complex than in the constant-flow-perfused rat heart. This is could be due to the over-ride of autoregulatory mechanisms involved in the control of coronary flow during reperfusion.

7.3 The effect of HO-mediated products in the constant-flow and constant-pressure-perfused rat heart.

HO-1-mediated breakdown of haem produces equimolar concentrations of CO, iron and bilirubin. Therefore, as HO-1 induction has been indicated as a protective factor in response to I/R under constant-flow conditions and at high constant-pressure levels, what role do the breakdown products of haem play in this response? In addition, does the absence of any of these factors explain the lack of protection observed in the constant-pressure system at 70 mmHg? In the constant-flow-perfused rat heart, the haemin-induced dilator response is likely to be produced as a result of CO release. In addition, the release of CO may act to potentiate the release of NO, as demonstrated by the increase in CPP seen upon treatment with L-NO-Arg. There is no direct evidence for the involvement of CO in the constant-pressure-perfused rat heart, as the techniques used to measure CO would be difficult to employ in conjunction with a perfused heart setup. Furthermore, as the half-life of CO is very short, this means that it is difficult to measure CO directly from tissue at the end of an experiment. However, as HO-1 expression and CO release have been shown to inhibit NOS and NO release, it is possible that the release of CO may have an inhibitory effect on NO release, thereby producing the constrictor action.

We hypothesize that HO-1 induction's protective response to I/R maybe due mainly to the activity of bilirubin, as ROS generation has been shown to be important in the development of ischaemia-induced tissue injury and contractile dysfunction. In the constant-flow rat heart, bilirubin production is increased,

which suggests a mechanism for the significantly improved recovery from I/R. This may be an important finding, as a number of previous studies have suggested that the protective effect of HO-1 induction is due to bilirubin release. However, bilirubin levels are not increased in the constant-pressure rat heart, where the degree of recovery from I/R is very high in both control and haemin-treated hearts, which may indicate that bilirubin is not important under these conditions. This is further demonstrated in the constant pressure system after an increased ischaemic insult of 30 min, where the recovery from I/R is completely abolished in both treatment groups, even though bilirubin levels are significantly increased in haemin-treated hearts. Therefore, it seems that the method of perfusion and pressure used in the experimental system is important in regulating the degree of protection conferred by bilirubin. To further investigate this effect, I would like to continue the study by perfusing both control and haemin pre-treated hearts with bilirubin, whilst exposing the tissue to 30 min I/R, and investigating whether recovery is improved under these circumstances.

The possible effect of iron in the response to haemin pre-treatment in the constant-pressure rat heart was also examined by using the iron chelator DFO. However, DFO had effects on both control and haemin-treated hearts. Interestingly, DFO increased CFR in both control and haemin-treated pre-ischaemic hearts in the constant-pressure experimental system. After I/R, iron removal improved recovery of cardiac function in control and haemin-treated hearts to a similar extent, suggesting that iron can be detrimental to the course of I/R injury. Therefore, the results of these experiments implicate CO, bilirubin and iron in the regulation of vascular tone, and in the prevention of I/R injury in the constant-flow- and constant-pressure-perfused rat heart. Further investigations to clarify these effects could involve the investigation of the vascular and post-ischaemic responses to exogenous treatment with CO and iron.

7.4. The effect of haemin pre-treatment in the control of vascular responsiveness in the perfused mesenteric vascular bed and kidney.

The effect of haemin-induced HO-1 expression on vascular tone was also determined in the perfused mesentery and kidney. What effect does haemin treatment have on the response to changes in vascular tone in these tissues? As it was difficult to determine haemin-induced HO-1 expression, the direct role of HO-1 in haemin-induced tissues was unclear. The main effect of haemin pre-treatment in the mesenteric bed was that it rendered prolonged vasoconstriction with PE more difficult to maintain. As there is no direct evidence for the involvement of HO-1 it can only be postulated that this effect may occur in response to increased release of CO if haemin increases HO-1 expression in this tissue. In addition, the vasodilator response to ACh in the presence of PE was also significantly reduced in the presence of haemin. Therefore, haemin pre-treatment (either through HO-1 induction or via a non-specific effect) appears to counteract the response to some of the vasoconstrictors investigated.

In the kidney, haemin pre-treatment did not affect the responses to vasodilators or to prolonged exposure to vasoconstriction after pre-contraction with 30 mM K^+. This was possibly due to the different mechanism of action utilised to induce an increase in basal tone. Nevertheless, in response to the vasoconstrictors ET-1 and PE, haemin pre-treatment produced a reduction in the peak height response compared with control. Therefore, haemin pre-treatment was found to aid the response to vasoconstrictors; this may be due to HO-1 induction and the possible release of CO, or to a further non-specific effect. Thus, to further investigate this effect in the kidney, I would like to investigate two other doses of haemin (lower and higher than 75 μmol/kg haemin), and also the effect of SnPP on vasodilator responses. In addition, it would be interesting to consider the effect of haemin pre-treatment in the constant-pressure-perfused rat kidney. Finally, I would also like to investigate the effect of the by-products of HO-1-induced haem catalysis: CO iron and bilirubin, on the vascular responsiveness of the rat kidney.

7.5. Final conclusion

In conclusion, the protective nature of HO-1 expression is as yet unclear. This study indicates that the protective nature of HO-1 is dependent both on the method of perfusion, and on the perfusion pressure used. These factors affect vascular responses to HO-1 expression, making it difficult to speculate on the effect of HO-1 in the intact human body. Such difficulties are also illustrated by the varying effects of haemin pre-treatment on vascular responsiveness in the perfused mesentery and kidney. Thus, the results of this study (and other published results) suggest that HO-1 has a very important yet complex role in the body. Finally, the further investigation of HO-1 in various pathological conditions of the human body offers an interesting challenge with the potential of great therapeutic rewards.

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